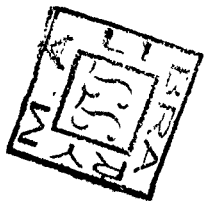


THERMODYNAMIC STUDIES OF THE DENATURATION OF OVALBUMIN

By

FAIZAN AHMAD

Abstract

A Tthesis submitted in fulfilment of the requirments for the degree of Doctor of Philosophy in Chemistry in the Faculty of Science of Aligarh Muslim University, Aligarh

1974

ABSTRACT

Ovalbumin is a major protein of egg white and contains 340 amino acid residues in a single polypeptide chain whose N- and C- terminal residues have been identified to be N-acetylated glycine and proline, respectively. It contains about three per cent carbohydrate which is linked through its aspartyl residues. An ovalbumin preparation showing size homogeneity usually exhibits charge heterogeneity due to the presence of three components, namely, A_1, A_2 and A_3 which are identical in gross molecular morphology and immunological activity but differ in phosphate content; A_1, A_2 and A_3 contain respectively two, one and no phosphate groups. Ovalbumin A_1 is the major fraction and forms about seventy five per cent of the total. Available data on intrinsic viscosity, second virial coefficient and frictional ratio suggest that the native ovalbumin is compact and globular. The native conformation of ovalbumin is partially disrupted by acid (below pH 3.5) and heat (above 50^0). Concentrated solutions of urea and guanidine hydrochloride caused more extensive unfolding of ovalbumin. The product of urea and guanidine hydrochloride denaturation showed marked tendency of aggregation and therefore, the protein transition showed limited reversibility. Pioneering researches

carried out in the laboratory of Kauzmann in the early fifties provided valuable clues to the mechanism of urea unfolding of ovalbumin. However, due to the irreversibility of the transition, thermodynamic study of urea or guanidine hydrochloride denaturation of ovalbumin could not be made. Further, the products of urea or guanidine hydrochloride denaturation of ovalbumin were not characterized in essential details.

Unlike ovalbumin, the guanidine hydrochloride denaturation of ovalbumin A_1 was found to be reversible under a variety of conditions of pH, temperature and guanidine hydrochloride concentration. As judged by the coincidence of transition measured by two independent properties, i.e., reduced viscosity and difference spectra as well as by the first order kinetics of the denaturation and renaturation process, it appears that the guanidine hydrochloride induced denaturation of ovalbumin A_1 involves one step. The product of guanidine hydrochloride denaturation of ovalbumin A_1 appears to be devoid of native structure. Furthermore, ovalbumin was found to be insoluble below 2 M guanidine hydrochloride whereas ovalbumin A_1 was soluble in the range, 0.2 - 7.0 M guanidine hydrochloride. These observations seemed very encouraging and prompted us to perform systematic studies on the kinetics and thermodynamics of guanidine hydrochloride induced denaturation of ovalbumin A_1.

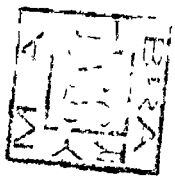
From the dependence of the equilibrium constant on the mean ion activity of guanidine hydrochloride, it was possible to conclude that ovalbumin A_1 binds about twelve molecules of guanidine hydro-

chloride more than the native one. These results do not permit unequivocal characterization of the binding sites on the protein for the denaturant. However, it is possible that aromatic amino acid residues particularly tyrosines may offer binding sites. Extrapolation of the free energy change for the ovalbumin A_1 denaturation to zero activity of guanidine hydrochloride yielded the free energy change, which is essentially the free energy of stabilization of the native protein conformation in aqueous solution; the value was found to be 6 Kcal $mole^{-1}$ at 25°. This suggested that the native conformation of ovalbumin A_1 is marginally more stable than the denatured one.

The guanidine hydrochloride induced denaturation of ovalbumin A_1 was sensitive to pH and the decrease in pH favoured unfolding. The pH dependence of the equilibrium constant for the guanidine hydrochloride denaturation of ovalbumin A_1 suggested that one carboxyl group is normalized during the unfolding process.

The guanidine hydrochloride induced transition was favoured by increase in temperature. Appropriate equations were obtained by the method of least squares to describe the observed temperature dependence of equilibrium constant for the denaturation of ovalbumin A_1. From the data, it was possible to calculate changes in enthalpy, entropy and heat capacity caused by the unfolding of ovalbumin A_1 by guanidine hydrochloride. Like other proteins, the guanidine hydrochloride denaturation of ovalbumin A_1 showed a large and positive change in heat capacity; the change in heat capacity was about

2,700 cal $mole^{-1}$ deg^{-1}. The large value of the change in heat capacity has been interpreted in terms of the unfavourable contacts of the hydrophobic moieties, which were 'buried' inside the native structure, to aqueous solvent.



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FAIZAN AHMAD

Date .....

Approved :

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(A. Salahuddin), Supervisor

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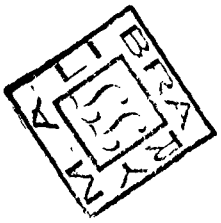

T1395

CERTIFICATE

I certify that work presented in the following pages has been carried out by Faizan Ahmad, and that it is suitable for the award of Ph.D. degree in Chemistry of the Aligarh Muslim University, Aligarh.

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I thank the Council of Scientific and Industrial Research (India) for financial assistance.

DEDICATION

Dedicated to my uncle and parents

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THERMODYNAMIC STUDIES OF THE DENATURATION OF OVALBUMIN

I. INTRODUCTION

Ovalbumin which is synthesized in oviduct comprises about three-fifths of the total proteins of egg white (1). It was first obtained in crystalline form by Hofmeister (2), and was named ovalbumin by Osborn and Campbell (3). It contains all the essential amino acids. The high content of lysine and methionine makes it nutritionally important protein. Some proteins which are devoid of any biological function in the native form, acquire catalytic function on denaturation. Native ovalbumin does not possess any catalytic function (4). However, on heat denaturation it is able to catalyse the decomposition of hydrogen peroxide (5). It is a glycoprotein. The carbohydrate moieties are linked with the protein polypeptide chain through its aspartyl residue (6). The carbohydrate units have been identified to be N-acetylglucosamine (5) and mannose (7). According to the recent data (8), 4 units of N-acetylglucosamine are linked with the aspartyl residue and 6 units of mannose are linked to C_4 of N-acetylglucosamine unit. Thus, the polymannosyl unit of ovalbumin is associated with the distal end of the N-acetylglucosamine unit.

Several lines of evidence suggest that ovalbumin consists of single polypeptide chain whose N- and C- terminal residues are

respectively N-acetylglycine (8) and proline (9). Although the amino acid compositions of ovalbumin obtained from different sources vary a little bit, the variations are generally too small to be of any experimental significance. The most recent data on amino acid composition of chicken ovalbumin are summarized in Table I. There are 71 aspartyl and glutamyl residues per 43,000 gram protein; the number of amide group is 30 ± 1 (11,12). Thus, the β- and γ- carboxyl groups are 41. The number of histidine, lysine and arginine residues are 6, 10 and 13, respectively. These findings are in good agreement with those reported earlier by Cannan et al. (13). Ovalbumin contains tyrosine residues, but none of them are available for titration (13,14) or accessible to chemical reagents (15,16). According to Weintraub and Schlanowitz (10) there are 8 half-cystine residues. However, it has recently been shown that ovalbumin contains 1 cystine and 4 cysteine residues (17,18) which would give a value of 6 for half-cystine residues. Ovalbumin contains 18 phenylalanine, 3 tryptophan and 14 methionine residues. The phosphate group seems to be linked with the serine residues of the protein (19). The amino acid sequence of ovalbumin has not been worked out so far. The partial N-terminal sequence of ovalbumin as deduced from the data of Thomson et al. (20), Milstein (21), Ottesen (22), and Ottesen and Wollenberger (23) is given in Figure 1. One of the striking features of the partial sequence of 54 amino acid residues is that the polypeptide is devoid of tyrosine residues.

Crystalline ovalbumin is homogeneous in ultracentrifuge (24)

TABLE I

AMINO ACID COMPOSITION OF OVALBUMIN[a]
(Molecular weight 43,000)

Amino Acid	No. of residues/ mole of protein[d]	Amino Acid	No. of residues/ mole of protein
Lysine	19	1/2 Cystine	8
Histidine	6	Valine	27
Arginine	13	Methionine	14
Aspartic Acid	28	Isoleucine	23
Threonine	14	Leucine	29
Serine	29	Tyrosine	10
Glutamic Acid	43	Phenylalanine	18
Proline	13	Tryptophan	3
Glycine	17	Alanine	32
		Amide N[b,c]	30 ± 1
		Total	346

[a] Data taken from reference 10
[b] Reference 11
[c] Reference 12
[d] The values have been rounded off to the nearest whole number

and on Sephadex column (25). However, the preparation showed charge heterogeneity as judged by its behaviour in (i) CM-cellulose column (26), (ii) starch gel electrophoresis (27), and (iii) polyacrylamide gel electrophoresis (25). Three components of ovalbumin, namely, A_1, A_2 and A_3 had been identified which differed in phosphate content; the components A_1, A_2 and A_3 contained two, one and zero phosphate groups respectively (28,29). Ovalbumin A_1* which is the major component may give rise to A_2 and A_3 by the progressive loss of phosphate group (30). The three components are immunologically indistinguishable (25,31), and have been shown to possess the same hydrodynamic behaviour (25). Apart from the phosphate content, a given ovalbumin preparation may have different genetic variants. Thus, Wiseman et al. (32) have recently recognized two genetic variants, namely, types A and B differing only in one amino acid residue; one asparagine residue in type A is replaced by aspartic acid in type B.

The molecular weight of ovalbumin, as measured by different physico-chemical procedures, has been reported in the range 40 - 46,000 (33-38); the most representative value being 43,000 (14).

The intrinsic viscosity of ovalbumin has been reported in the range 4.0 - 4.3 cc/g (39-43). Other physico-chemical parameters are listed in Table II. Thus, the data of Table II suggest that the native ovalbumin is a globular and compact molecule. It contains about 39% α-helix and 27% β-structure (49). The intrinsic viscosity of Oval A_1 determined as a part of this study

* Throughout this thesis ovalbumin A_1 will be abbreviated Oval A_1.

THE PARTIAL AMINO ACID SEQUENCE OF OVALBUMIN

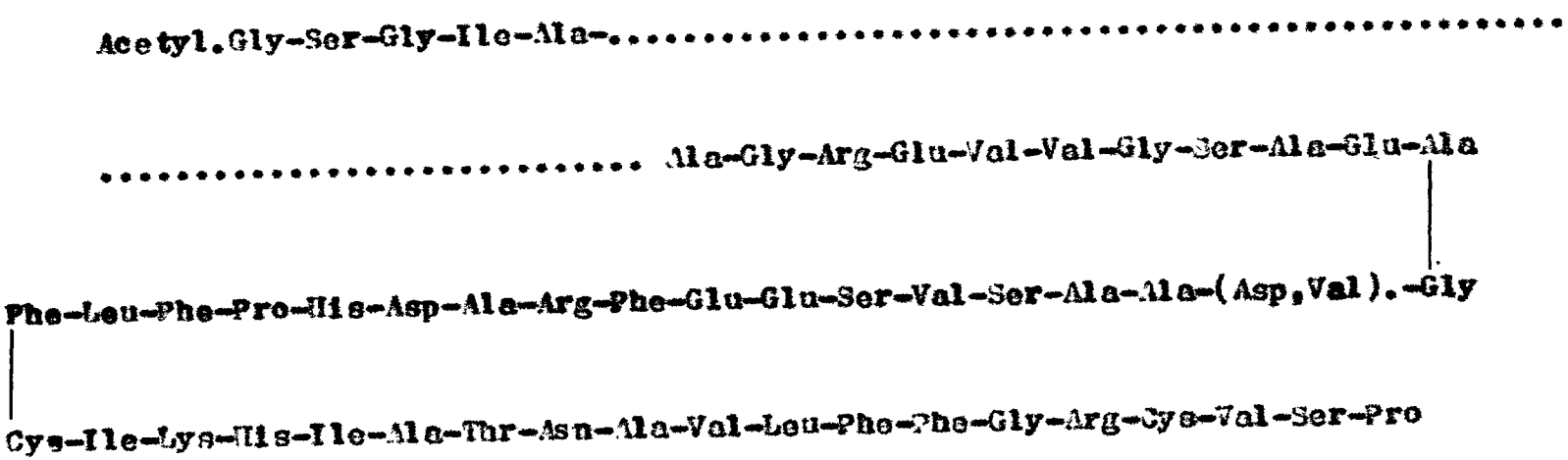

Figure 1. Based on the results of Thompson et al. (20), Milstein (21), Ottesen (22), and Ottesen and Wollenberger (23).

was 3.9 cc/g which is comparable to that reported for ovalbumin. Further, the elution behaviours of ovalbumin and Oval A_1 on Sephadex column were found to be exactly identical. These facts strongly suggest that the gross molecular morphology of Oval A_1 is similar to that of ovalbumin.

The native conformation of ovalbumin seems to be sensitive to pH at lower pH values. However, there is controversy about the pH for the onset of acid denaturation of ovalbumin. According to Bull and Breese (50), the pH for the onset of denaturation is about 3.0 which was confirmed later by Ottesen and Wallevik (51). Yang and Foster (52) found that the optical rotation of ovalbumin remains unchanged on lowering the pH to 3.0. Strikingly, Glazer et al. (53) noticed no change in absorption spectra of ovalbumin in the pH range 3 - 9. Light absorption study of Ansari of this laboratory (54), however, indicated definite change in optical properties of ovalbumin when pH was lowered below 3.0. The reduced viscosity of Oval A_1 did not change on decreasing the pH from 7.0 to 3.5. Further decrease in pH below 3.5 produced 2.5-fold increase in reduced viscosity of the protein at pH 1.0 suggesting an appreciable conformational change caused by acid.

That ovalbumin undergoes thermal denaturation at lower pH values, is supported by several observations reported earlier. Thus, heating of ovalbumin solution of pH 3.0 at 55° for 90 minutes produced 95% denaturation (55). Oval A_1 in phosphate buffer, pH 7.0, ionic strength 0.15, is resistant to heat up to 50°, as judged

TABLE II

PHYSICAL PARAMETERS OF THE NATIVE AND DENATURED OVALBUMIN

Experimental condition	$[\eta]$	$E^{1\%}_{1cm}$	$s_{20,w}$	f/f_o	a_o	b_o
Native	4.0–4.3[a–c]	7.37	3.5–3.6	1.10–1.20	-170°	-142°
6 M Gdn.HCl, 25°	31.6[f]	7.54	-	-	-544°	+ 19°
6 M Gdn.HCl plus β-mercaptoethanol, 25°.	34.3[f]	-	2.18[f]	-	-540°	+ 33°
8 M urea, pH 2.0, 25°.	30.2[g]	-	-	-	-	-

The value of $E^{1\%}_{1cm}$, a_o and b_o were taken from reference 44, and those of f/f_o were taken from reference 40, 45–47.
[a–c] References 39–43. [f] Reference 44. [g] Reference 48.

by viscosity measurements; increase in temperature beyond 50° caused thermal denaturation. It is to be noted with interest that the thermal denaturation of Ovalbumin has been recently shown by Moyer (56) to follow first order kinetics. Native conformation of ovalbumin is altered significantly by organic acids such as fatty acids; the effectivity increases with the chain length (50). The cationic detergents such as dodecyldimethylbenzyl-ammonium chloride, dodecyltrimethyl and dodecyl-ammonium chloride, and acetyldimethylphenyl-ethylammonium bromide, denatured ovalbumin rapidly at room temperature (57). However, the products of such denaturation are usually aggregates and hence could not be uniquely characterized. Recent studies on urea denaturation have revealed that most proteins exist as random coils in 8 - 10 M urea (58,59). Ovalbumin undergoes extensive denaturation in 8.6 M urea (60). The native ovalbumin is however, resistant to 1 - 6 M urea at 30° (61). Frensdroff *et al.* (62) have measured the intrinsic viscosity of ovalbumin in 10 M, pH 7.0 maintained by phosphate buffer and found a value of 34 cc/g at 35°. It should be noted that the tendency of aggregation of ovalbumin in 10 M urea is very pronounced, and therefore, it seems likely that the value of intrinsic viscosity measured by Frensdroff *et al.* represents an overestimation. Aggregation of the denatured ovalbumin is considerably reduced at lower pH. The intrinsic viscosity of ovalbumin in 9 M urea plus β-mercaptoethanol (pH 3.0, ionic strength 0.15) was determined to be 29.2 cc/g (48) which is what one should have expected from the

empirical equation of Lapanje (59) for linear randomly coiled proteins. The urea denaturation of ovalbumin was extensively investigated by Simpson and Kauzmann (63) who found limited renaturation of ovalbumin previously denatured by urea under different conditions of temperature, pressure and pH. They concluded that the urea denaturation of ovalbumin involves more than one step under various conditions of temperature, pH and pressure, and that the protein denaturation involves unfolding followed by aggregation. Presumably it is the latter which makes the urea denaturation predominently an irreversible process. From the kinetic studies of urea denaturation of ovalbumin Suzuki (64) was able to determine the various parameters such as enthalpy and heat capacity change for the activation process. The heat capacity change for the activation of the native ovalbumin molecule to the activated state was found to be independent of temperature, and was 2,000 cal $mole^{-1}deg^{-1}$. Simpson and Kauzmann (63) determined a value of 1,800 cal $mole^{-1}deg^{-1}$ from their kinetic results on urea denaturation of ovalbumin. Considering the inherent uncertainty in the determination of heat capacity change from kinetic or equilibrium results, the two values for the heat capacity change are in good aggreement. The change in heat capacity for Gdn.HCl denaturation of Oval A_1 as determined in this study was about 40% higher than the change in heat capacity for activation deduced from kinetic data on urea denaturation of the whole ovalbumin (63,64). It should be noted that the denatured taka-amylase A has considerably higher heat capacity than the activated protein (65). This is understandable because the exposure

of hydrophobic groups due to protein unfolding would be incomplete in the activated state.

Proteins generally lose all the elements of their native conformation in 6 M Gdn.HCl (66-70). Holt and Creeth (44) have recently determined the intrinsic viscosity of ovalbumin in 6 M Gdn.HCl in absence and presence of β-mercaptoethanol, and the respective values of intrinsic viscosity were 31 and 34.6 cc/g. Lapanje (71) has shown that ovalbumin behaves as a randomly coiled protein in 4.5 M guanidinium thiocyanate at 25°, the intrinsic viscosity was 30.3 cc/g. The intrinsic viscosity of Oval A_1 in 6 M Gdn.HCl containing β-mercaptoethanol at 25° was measured to be 31 cc/g. This by implication would mean that Oval A_1 behaves as a random coil in 6 M Gdn.HCl.

The kinetics of Gdn.HCl denaturation of ovalbumin was studied by Schellman et al. (73). The important conclusions of their studies are that Gdn.HCl denaturation of ovalbumin is similar to the urea denaturation and involves more than one step, and that the denatured protein does not renature below 2 M Gdn.HCl. Recently Holt and Creeth (44) have also reported limited reversibility of Gdn.HCl denaturation of ovalbumin. Our preliminary studies with whole ovalbumin confirmed the last conclusion (73).

From what has been described above, it follows that the native conformation of ovalbumin is sensitive to the well known perturbants such as acid, heat, urea and Gdn.HCl, and that none of these denaturations have been treated quantitatively so far.

Thermodynamic analysis of the data hitherto obtained on the denaturation of ovalbumin, has not been feasible primarily because the protein unfolding was found to exhibit very limited reversibility. The denatured protein showed marked tendency of aggregation which made the interpretation uncertain. The product of denaturation could not be characterized in terms of well defined physico-chemical parameters. Furthermore, the ovalbumin preparations used in earlier studies were of doubtful purity. Even if ovalbumin preparation is homogeneous with respect to size, it does contain at least three different fractions, A_1, A_2 and A_3. It is possible that the three components may adopt different pathways for the unfolding. This would make the first order kinetic plot for ovalbumin denaturation non-linear as has been actually observed for urea and Gdn.HCl denaturation of ovalbumin. This aspect of the problem will be investigated in this laboratory in the near future.

Contrary to the behaviour of ovalbumin, the Gdn.HCl denaturation of Oval A_1 was found to be completely reversible under a variety of conditions of Gdn.HCl concentration, pH and temperature. From the nature of the coincidence of the transitions measured by two independent properties, i.e., reduced viscosity and difference spectra, as well as from the kinetic results, the transition of Oval A_1 appears to involve one step. The product of Gdn.HCl denaturation of Oval A_1 did not show any detectable aggregation and was devoid of any non-covalent structure. Interestingly we noticed that the whole ovalbumin is insoluble below 2 M Gdn.HCl, whereas, Oval A_1 was soluble throughout the entire range of the denaturant concen-

tration (0.2 - 7.0 M). In view of these interesting findings we thought it worthwhile to perform systematic studies on the Gdn.HCl denaturation of Oval A_1. Such studies would be essential for a proper understanding of the thermodynamic stability of one of the most important proteins of egg white under native conditions. Further, the thermodynamic parameters for the Gdn.HCl induced conformational transition will hopefully provide valuable informations regarding the nature of different non-covalent interactions which stabilize the native protein conformation.

II. EXPERIMENTAL

A. Materials

1. Preparation of Egg White Oval A_1.

Ovalbumin used in these studies was isolated from egg white according to the method of Kekwick and Cannan (74). The membrane of egg white, separated from fresh eggs, was ruptured by the help of a homogenizer (frothing was avoided). To this homogenate, was added equal volume of 36% (w/v)* sodium sulphate solution gradually and with continuous stirring. This mixture was kept for 2 hours in order to precipitate out globulins. The mixture was centrifuged, and the reddish-yellow coloured filtrate was separated from the precipitate. To the filtrate, was added 0.2 M sulphuric acid to bring the pH to 4.8, and then solid sodium sulphate was added with continuous stirring till precipitate began to be formed. Sodium sulphate, if in excess, was removed by decantation. The mixture was kept for 18 hours at room temperature. The precipitate (crude ovalbumin) was separated by centrifugation and dissolved in water. Again sodium sulphate crystals were added to the solution with

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* The concentrations will throughout be expressed in gram solute/ 100 ml solution (w/v), unless otherwise stated.

continuous stirring till the precipitate of ovalbumin started developing, and the mixture was allowed to stand for 18 hours. The crystallized ovalbumin was separated from its filtrate by centrifugation. The process of recrystallization was repeated 4 times and the final precipitate of ovalbumin containing salt was dried in a vacuum desiccator.

As shown in Figure 2, the contamination of ovalbumin with other egg white proteins was checked on CM-cellulose column chromatography according to the method of Rhodes et al. (26).

Routinely, ovalbumin preparation which was extensively dialysed against water was stirred gently for about 30 minutes with CM-cellulose (approximately 5 grams adsorbant per gram protein), which was previously equilibrated with ammonium acetate buffer, pH 4.0, ionic strength 0.025. The suspension was filtered and the residue was washed with ammonium acetate buffer, pH 4.5, ionic strength 0.025, on a Buchner funnel. Oval A_1 was then eluted by treating CM-cellulose containing protein with ammonium acetate buffer, pH 4.6, ionic strength 0.025. This suspension was filtered and the filtrate was concentrated.

2. Proteins.

Pancreatic ribonuclease, Type II-A; bovine serum albumin, Type F; and egg white lysozyme were purchased from Sigma Chemical Company (U.S.A.). Salt free myoglobin (crystallized and lyophilized) was obtained from Mann Research Laboratory, Inc., U.S.A. Chymotryp-

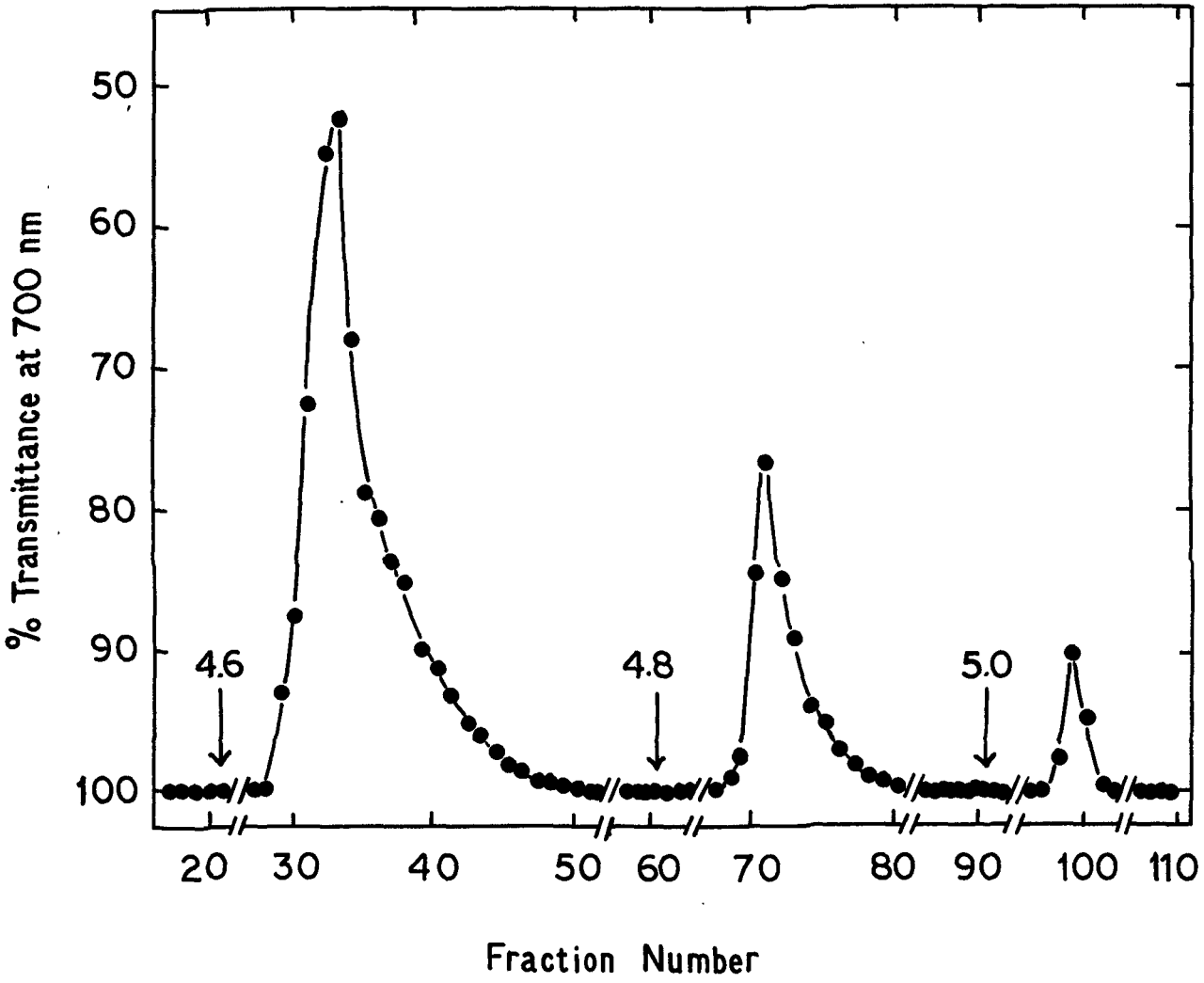

Figure 2. Carboxymethyl-Cellulose Column Chromatography of Ovalbumin Isolated from Egg White. Experimental conditions: 120 mg of 5 x crystallized ovalbumin in 200 ml ammonium acetate buffer (pH 4.0, ionic strength 0.025) was applied on the column (2.1 x 18 cm) and eluted in 10 ml fractions with a flow rate of 75 ml/hr with the three ammonium acetate buffers shown by arrows.

sinogen A and pepsinogen were from Worthington Biochemical Corporation (U.S.A.)and Nutritional Biochemical Corporation (U.S.A.), respectively. Lyophilised preparation of papain which gave a single band on polyacrylamide gel electrophoresis (75), was from V.P. Chest Institute, Delhi.

3. Guanidine Hydrochloride.

Guanidine hydrochloride (Gdn.HCl) used in these studies was either obtained from Nutritional Biochemical Corporation, U.S.A., or it was prepared from guanidinium carbonate according to the method essentially due to Nozaki and Tanford (76).

The commercial sample of Gdn.HCl was purified before use. The purification procedure involved washing of the sample with cold distilled acetone on a Buchner funnel. Acetone treated Gdn.HCl was dissolved in a minimum volume of distilled ethanol containing activated charcoal at 40°, and was filtered. The filtered solution was kept in cold for crystallization. Recrystallized Gdn.HCl, separated from mother liquor, was dissolved in warm water at 40° to get saturated solution. This solution was again kept in cold, and crystals of Gdn.HCl were separated and dried by blowing hot air.

Preparation of Gdn.HCl from guanidinium carbonate involved the following procedure. About 500 grams of guanidinium carbonate, washed with distilled acetone, was dissolved in 1 litre of water at 40°. The solution was treated with activated charcoal, and was filtered. To the aqueous solution of guanidinium carbonate, was

added 1.5 litre of distilled ethanol, and the mixture was kept overnight in cold before it was filtered. The crystalline powder of guanidinium carbonate was collected on a Buchner funnel and was rinsed with 50% (v/v) chilled ethanol-water mixture and then with ethanol. The yield was 88%.

A slurry containing 440 grams of crystallized guanidinium carbonate and 250 ml of distilled water was cooled with ice. To this was added 20% constant boiling hydrochloric acid with continuous stirring till pH became 4.0. It was allowed to stand overnight and the pH readjusted to pH 4.0. A portion of this mixture was flash-evaporated at 40° until crystallization set in. It was stored in cold and the crystalline mass was filtered. To the mother liquor, was added the Gdn.HCl solution of pH 4.0 and was flash-evaporated until crystallization started. This procedure was repeated until 300 grams of guanidine hydrochloride was obtained. Finally, crystals of Gdn.HCl were recrystallized from water. A 50% of Gdn.HCl aqueous solution was prepared and flash-evaporated at 40°. The flash-evaporated mixture was kept in cold and the recrystallized Gdn.HCl was collected and dried in vacuum desiccator. The melting point was checked and was found to be in the expected range 185-186°; reported value lies in the range 187-188° (77). A 6 M Gdn.HCl solution had an absorbance less than 0.15 at 225 nm.

4. Urea.

Urea obtained from BDH (India) was recrystallized twice from

50% (v/v) ethanol-water mixture at 40°. The purified urea was dried by blowing hot air.

5. Hydrochloric Acid.

A constant boiling hydrochloric acid was prepared according to the method of Foulk and Hollingsworth (78). Analytical reagent grade hydrochloric acid obtained from BDH (India) was diluted to specific gravity of about 1.1 by the addition of distilled water. The mixture was distilled at a rate of 8-10 ml per minute. About 75% of the original volume was distilled and discarded, and the next 15% distillate consisting of constant boiling hydrochloric acid was collected. The pressure was recorded by Fortin's barometer at the begining as well as at the end of distillation and was 738.71 and 741.40 mm, respectively. The concentration of constant boiling hydrochloric acid corresponding to the mean value of a pressure of 740.05 mm was read from the plot between the weight of distillate containing 1 mole of hydrochloric acid and atmospheric pressure which was obtained from the data of Foulk and Hollingsworth.

6. Ion-Exchangers.

Carboxymethyl-cellulose was purchased from Calbiochem, Los Angeles, U.S.A. The ion-exchange resins, Dowex 50W-X8 (20-50 mesh) in sodium form and Dowex 1W-X8 (20-50 mesh) in chloride form, were obtained from J.T. Baker Chemical Company (U.S.A.). The anion exchange resins in hydroxyl and acetate cycles were prepared from

Dowex 1W-X8, whereas, the resins in hydrogen and ammonium cycles were obtained from Dowex 50W-X8. The deionized protein solution was obtained by passing the extensively dialysed protein solution through a mixed bed column, prepared according to Dintzis (79).

7. Other Materials.

Dialysor tubings and millipore filters HAWP 01300 (pore size 0.45 micron) were obtained respectively from Arthur Thomas Company, Philadelphia, U.S.A., and Millipore Corporation, U.S.A. β-Mercapto-ethanol was from Sigma Chemical Company, U.S.A. Potassium hydrogen phthalate as well as sodium tetraborate, purchased from B.D.H. (India), were first recrystallised and then used for preparation of the standard buffer. Distilled acetone and ethanol were used. Other chemicals which were of reagent grade were acetic acid, ammonium acetate, ammonium chloride, barium hydroxide, bromine, copper sulphate, disodium hydrogen phthalate, lithium sulphate, phosphoric acid, potassium chloride, sodium acetate, sodium azide, sodium bicarbonate, sodium carbonate, sodium chloride, sodium dihydrogen phosphate, sodium hydroxide, sodium molybdate, sodium potassium tartrate, sodium sulphate and sodium tungstate.

All glass double distilled water was used throughout these studies.

B. Methods

1. Determination of Protein Concentration.

(a) Dry weight method.

For all proteins, except lysozyme and papain, relatively concentrated aqueous solutions were prepared, which were extensively dialysed against several changes of water in cold, each after an interval of about half an hour. Traces of insoluble material, if any, were removed either by centrifugation at 10,000 revolutions per minute or by filtration through millipore filter. A clear dialysed protein solution was passed through Dintzis column (79) to get isoionic preparation of the protein. The isoionic pH of about 1% Oval A_1 was determined to be $4.9 \pm 0.0_4$. A fixed volume of distilled water equal to that of protein solution containing about 15 mg protein was taken in four well cleaned weighing bottles and heated at 107° to constant weights. The isoionic preparation of the protein was then taken in the weighing bottles by weight and dried to constant weights in the oven at 107°. In case of lysozyme, protein was dissolved in 1.0 M potassium chloride solution and dialysed against several changes of 0.1 M potassium chloride. The protein solution and the dialysate were separately heated to constant weight, and the protein concentration was determined by the difference.

(b) Lowry's method.

The column was monitored by Lowry's method (80).

Following Folin and Ciocalteu (81), 100 g of sodium tungstate, 50 ml of 85% of phosphoric acid along 700 ml of water were transferred to a 2 litre flask fitted with reflux condenser and placed on a heating plate. The solution was refluxed, and to it were added 150 g of lithium sulphate, 50 ml of water and few drops of liquid bromine. The resulting mixture was boiled without condenser for about 15 minutes to drive off excess bromine. This mixture was then cooled and diluted to one litre. The solution, known as Folin-phenol reagent, was then stored in a coloured bottle.

The copper reagent was prepared by taking 2% sodium carbonate in 0.1 N sodium hydroxide (A), 2% potassium tartrate solution (B) and 1% copper sulphate solution (C) in the ratio of 100:1:1 (v/v/v). It is important that different solutions should be mixed in the sequence A, B, C.

To a requisite volume of protein solution, 5 ml of copper reagent was added, and after 10 minutes 0.5 ml of 50% (v/v) diluted Folin-phenol reagent was added. The optical density of the resulting solution containing protein in the range 10-200 microgram was measured at 700 nm after 30 minutes on a CarlZeiss Jena Spectrocolorimeter, Spekol, using glass cuvettes of 1 cm light path.

2. Measurement of pH.

Elico pH meter model LI-10 in conjunction with glass and

calomal electrodes was used for pH measurements at room temperature. The pH meter was standardized using 0.05 M phthalate buffer, pH 4.01, and 0.01 M borate buffer, pH 9.0 at 25°. The pH of each solution was determined after viscosity or spectral measurements.

3. Chromatography of Oval A_1 on CM-Cellulose Column.

Carboxymethyl-cellulose powder was first suspended and allowed to swell for about 5 hours. The large and fine particles were removed by decantation. In order to convert CM-cellulose to sodium cycle, it was treated with 0.1 N sodium hydroxide and washed with excess water till CM-cellulose became free from alkali. The resin was then converted to hydrogen cycle by treatment with 0.1 N hydrochloric acid with several changes. It was washed with excess of water to remove hydrochloric acid. Carboxymethyl-cellulose in hydrogen cycle was suspended in ammonium acetate buffer, pH 4.0, ionic strength 0.025, for at least one hour. The slurry was poured into the column half filled with the equilibrating buffer. It is important that slurry should neither be too thick nor too dilute. Every care was taken to ensure that the packed column was free from air bubbles.

About 200 mg of Oval A_1, prepared by batch-wise fractionation with CM-cellulose, was applied on the column. The protein was eluted in 10 ml fractions with ammonium acetate buffer, pH 4.6, ionic strength 0.025, at a flow rate of 75 ml/hour (see Figure 3). Then two buffers of higher pH namely, pH 4.8 and 5.0, were passed to

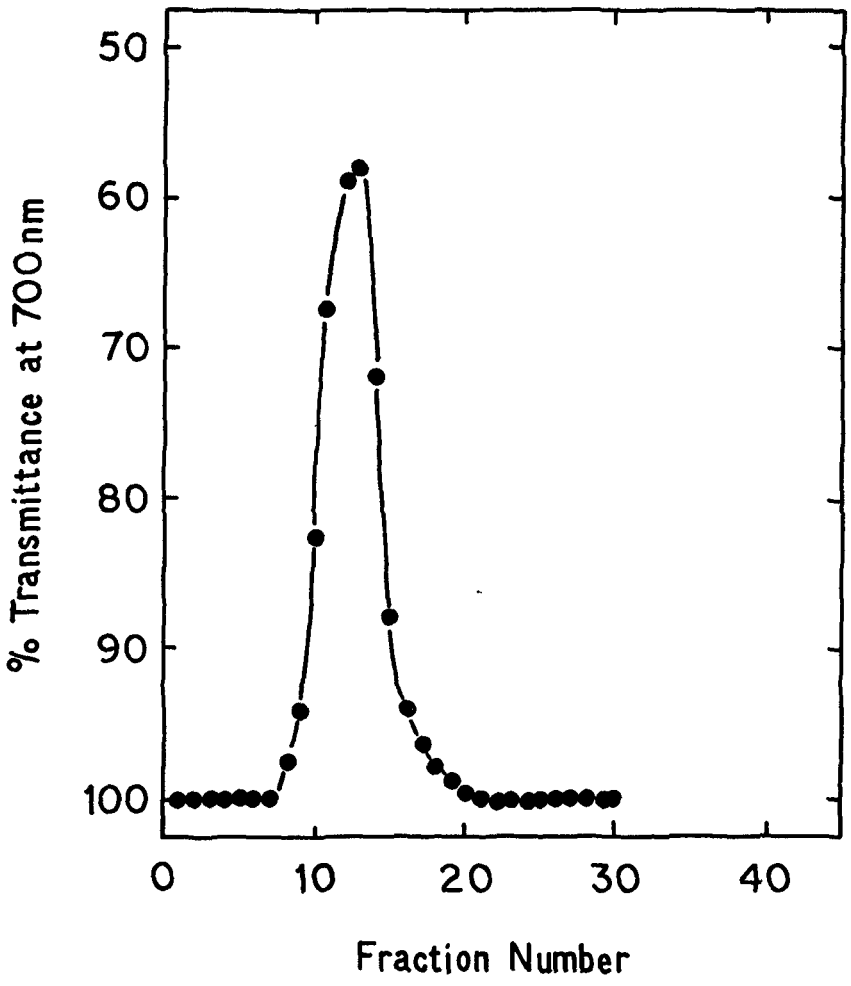

Figure 3. Chromatography of Oval A_1 on CM-Cellulose Column. Experimental conditions: The same as in Figure 2 except that the sample size was 200 mg in 10 ml and column size was 2.1 x 11 cm.

check the elution of protein; no protein was eluted.

4. Measurement of Viscosity.

Cannon-Fenske type capillary viscometers with flow times of the order of 400 seconds for 5 ml water at 25°C were used in most of the measurements. Ostwald type viscometers with flow times more than 60 seconds were also used in the kinetic measurements on occasions.

(a) Washing of viscometer.

It was noted that improper washing and drying of viscometers led to serious errors in results. Therefore, extreme care was taken in washing and drying the viscometer. The new viscometer was filled with chromic acid followed by nitrating mixture. It is important that the viscometer filled with nitrating mixture should not be kept for longer time, because yellow substance got adsorbed on the capillary surface. After removing the nitrating mixture, viscometer was washed with filtered distilled water, and then dried by passing dust free air. After viscosity measurement, the protein solution was removed from the viscometer. Viscometer was then filled with clear dilute detergent solution and kept for some times. It was washed with water and dried in the same way. When not in use, the viscometer should be cleaned and dried.

(b) Measurement.

Solvents as well as protein solutions were filtered through millipore filters. Filtered solution was taken in a pipette and delivered to the vertically clamped viscometer kept in a well insulated glass tank whose temperature was regulated by circulating water from an ultrathermostat (MeO Thermostat, Hungary). Flow time for solvent (t_o) and that for protein solution (t) were measured by the help of a stopwatch with a least count of 0.1 second. A minimum of five measurements of flow time per solution were made; the deviation was generally less than 0.2 second.

The intrinsic viscosity $[\eta]$, is calculated in the following manner (83).

$$[\eta] = \lim_{C \to 0} \eta_R = \lim_{C \to 0} \left(\frac{\eta - \eta_o}{\eta_o C} \right)$$

$$= \lim_{C \to 0} \left(\frac{t - t_o}{t_o C} \right) + \frac{1 - \bar{v}_2 \rho_o}{\rho_o}, \qquad (1)$$

where C is the protein concentration in g/ml, η_R is the reduced viscosity, η is viscosity of protein solution, η_o is viscosity of solvent, $\bar{v}_2$ is the partial specific volume of protein, and ρ_o is the density of the solvent which was measured by weighing known volume of the solvent.

The quantity $\left(\frac{t - t_o}{t_o C} \right) + \left(\frac{1 - \bar{v}_2 \rho_o}{\rho_o} \right)$ presented in

Equation 1 very closely approximates the reduced viscosity. This quantity was plotted against protein concentration and extrapolation was made according to the equation (68),

$$\eta_R = [\eta] + k'[\eta]^2 c, \qquad (2)$$

where k' is Huggins constant.

6. Measurements of Optical Density.

(a) Measurement of extinction coefficient.

All measurements were made on solutions which had been previously filtered through millipore filters. The extinction coefficient of Oval A_1 in phosphate buffer, pH 7.0, ionic strength 0.20 was determined using CarlZeiss Spectrophotometer, VSU2-P, with quartz cuvettes of 0.999 cm light path. Different amounts of isoionic protein stock solution, whose concentration was previously determined by dry weight method, were weighed in 5 ml standard flasks. Ionic strength of the protein solution was maintained by the addition of the required amount of sodium chloride. The optical density of 10 protein solutions of varying concentration were measured at room temperature and at different wavelengths. The extinction coefficient, $E^{1\%}_{1cm}$, at each wavelength was computed from the slope of the straight line between optical density and protein concentration expressed in g/100 ml. The straight line was obtained by the method of least squares. The values of $E^{1\%}_{1cm}$ with its wave-

length in parentheses were found to be 12.413 ± 0.010 (242 nm), 5.113 ± 0.035 (257.5 nm), 6.190 ± 0.011 (265 nm), 7.150 ± 0.005 (280 nm), 4.446 ± 0.004 (288 nm) and 2.92 ± 0.007 (293 nm).

(b) Measurement of difference spectra.

The change in absorption spectrum of the native protein solution upon denaturation was recorded on a Beckman model DK-2A ratio recording spectrophotometer equipped with a thermostatically controlled cells holder whose temperature was regulated within ± 0.1° by circulating water at 25°. The difference spectrum of Oval A_1 in 6 M Gdn.HCl solution against the native protein in phosphate buffer, pH 7.0, ionic strength 0.20 in the range 320 - 250 320 - 250 nm was measured. As shown in Figure 4, the main features of the difference spectrum are the maxima at wavelengths 293, 288, 265, 257.5 and 242 nm. The first three wavelengths were selected for the present denaturation studies. The optical density of protein solutions containing varying concentrations of Gdn.HCl were recorded. The absorption of protein solution at a particular Gdn.HCl concentration, at each wavelength, was divided by the protein concentration in g/100 ml to give $E^{1\%}_{1cm}$ which was subtracted from the corresponding $E^{1\%}_{1cm}$ of the native protein to obtain the value of $\Delta E^{1\%}_{1cm}$. The results are expressed in terms of $\Delta E^{1\%}_{1cm}$ as a function Gdn.HCl concentration.

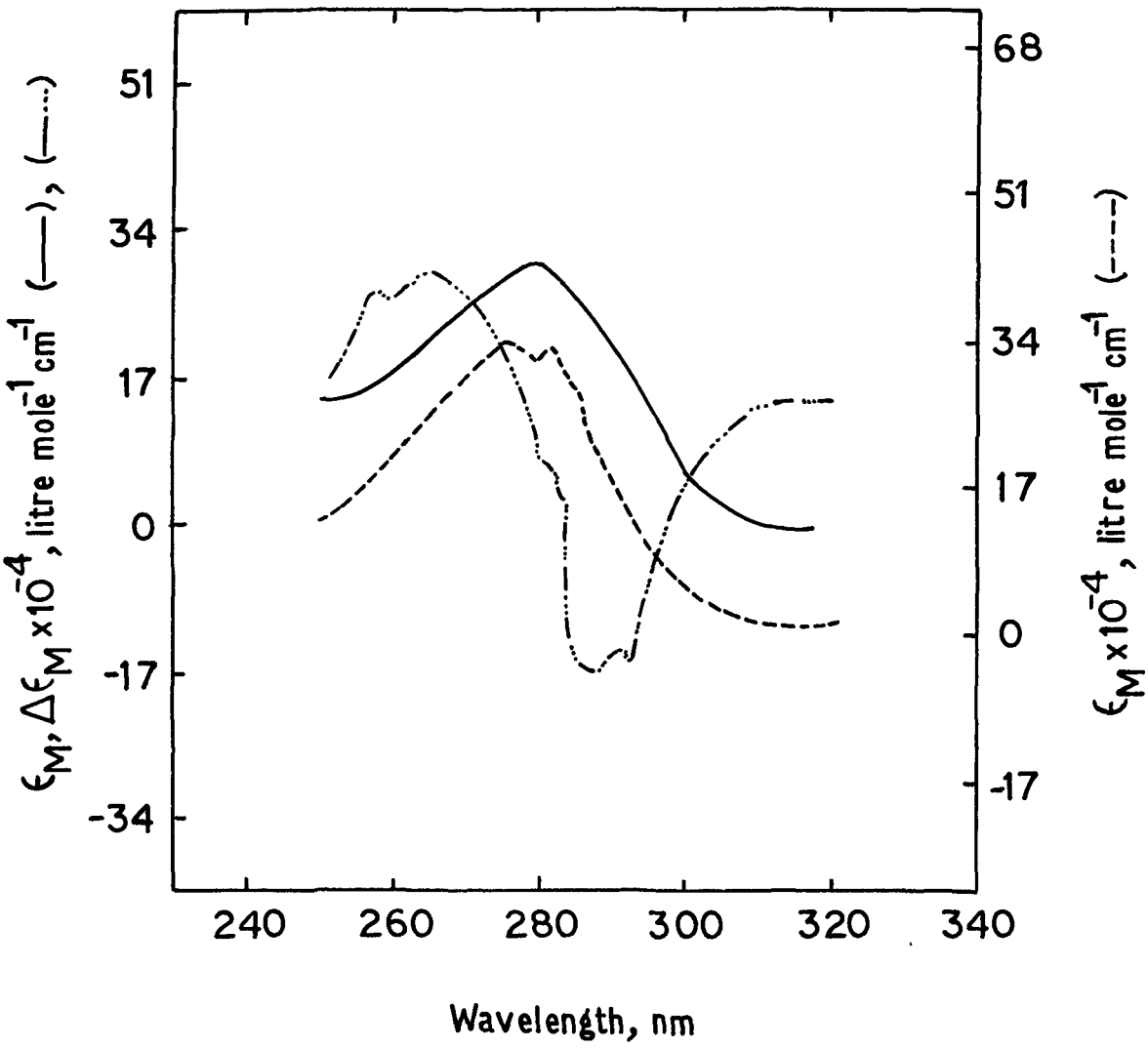

Figure 4. Effect of Gdn.HCl Concentration on the Absorption Spectrum of Oval A_1. Experimental conditions: (———) Oval A_1 in phosphate buffer, pH 7.0, ionic strength 0.15; (- - -) Oval A_1 in 6 M Gdn.HCl, pH 6.8; (—— ... —— ... ——) the difference spectrum with native Oval A_1 in the reference and the denatured one in the sample beam.

7. Denaturation Experiment.

Protein stock solution whose concentration was previously determined by dry weight method was weighed in 5 ml volumetric flask. Protein solution was diluted with water followed by the addition of solid disodium phosphate and sodium hydrogen phosphate required for maintaining a constant pH of 7.0, ionic strength 0.15. Then desired amount of Gdn.HCl was added by weight. The final volume was made up to 5 ml. All solutions were prepared at room temperature. The measurements of viscosity and $\Delta E^{1\%}_{1cm}$ on solutions were usually made 10 hours after the preparation.

8. Renaturation Experiment.

Solutions for renaturation studies were prepared in the same way as those for denaturation experiments except that in this case, protein solution was first exposed to 7 M Gdn.HCl for about 15 minutes and diluted with water to 5 ml. The kinetic experiments to be described later indicated that the denaturation is complete within 9 minutes at 3.1 M Gdn.HCl concentration. Therefore, the treatment of protein with 7 M Gdn.HCl for 15 minutes was obviously more than sufficient for attainment of equilibrium.

9. Effect of pH on Guanidine Hydrochloride Denaturation.

The pH induced transition of Oval A_1 was studied in the pH range 1 - 7, by measuring viscosity of 0.38% protein solution

containing fixed amount of Gdn.HCl as a function of pH. Protein stock solution and Gdn.HCl were taken by weight and the pH was adjusted by adding appropriate volumes of hydrochloric acid or sodium hydroxide. The transition induced by lowering the pH was found to be reversible at all Gdn.HCl concentration. The reversibility was checked by measuring the viscosity of protein solution containing Gdn.HCl at any pH obtained in going from neutral to acid pH or vice-versa.

10. Effect of Temperature on Guanidine Hydrochloride Denaturation.

The effect of temperature on the denaturation equilibrium of Oval A_1 at different Gdn.HCl concentrations was investigated in the temperature range 10-60°. Protein solutions of pH 7.0, containing a fixed amount of Gdn.HCl were prepared at room temperature. The protein concentration was 0.38%. Filtered solution was taken in a viscometer and the flow time determined at each temperature after a period which was sufficient for the attainment of equilibrium. The time required for temperature equilibration was found to be less than 30 minutes. In order to test whether the thermally induced Gdn.HCl denaturation is reversible, the protein solution at 60° was cooled down gradually to the lower desired temperature and the viscosity measured. The agreement between the viscosity at the lower temperature in the forward and reverse run was excellent.

11. Kinetics of Denaturation and Renaturation.

All kinetic measurements were made at 25°. The progress of the denaturation or renaturation reaction was followed by measuring the viscosity of the protein (0.38%) solution at a fixed concentration of Gdn.HCl. Solutions for the kinetics of denaturation and renaturation studies were made in the same way as described above. For kinetics of denaturation, the moment Gdn.HCl was added to the protein solution a stopwatch was started. Protein solution was taken in a viscometer already clamped in water tank at 25°. The first stopwatch was stopped and the second started to record the time of fall in the viscometer. Hence the time of reaction was the sum of readings on both the stopwatches. The procedure for the kinetics of renaturation studies was exactly similar except that the time was recorded from the moment protein solution, which was previously exposed to concentrated Gdn.HCl solution, was diluted by adding buffer. The reaction could be followed beginning at 1 minute after mixing. The results were plotted in the form of plot of $\ln \left| \eta_{R_{t=t}} - \eta_{R_{t=\infty}} \right|$ versus $t/t_{0.5}$. Experiments on kinetics of denaturation and renaturation were carried out in the range of Gdn.HCl concentration 1.9 - 3.1 M and at various pH values. The measurement on the kinetics of denaturation and renaturation were also made at different temperatures.

III. EXPERIMENTAL RESULTS

A. The Native State of Oval A_1

Oval A_1 in phosphate buffer, pH 7.0, ionic strength 0.15, at 25° was taken to represent the native state of the protein. The intrinsic viscosity of the protein under these conditions was determined to be 3.85 ± 0.13 cc/g (see Figure 5) which is about 6% lower than the values for the whole ovalbumin, reported in the earlier literature; the reported values lie in the range 4.0 - 4.3 cc/g (33,43). Thus, the intrinsic viscosity of Oval A_1 lies in the range 3 - 4 cc/g expected for globular native proteins (58).

Intrinsic viscosity of the protein depends on the shape and hydration of the molecule. This statement is described by the expression (24),

$$[\eta] = \nu(\bar{v}_2 + \Sigma \delta_i v_i^0), \qquad (3)$$

where ν is shape or Simha factor, and is 2.5 for suspended sphere (24) and larger for ellipsiods; $\bar{v}_2$ and v_i^0 represent the partial specific volumes of protein and the ith solvent component, respectively; δ_i is the gram of the ith component bound to one

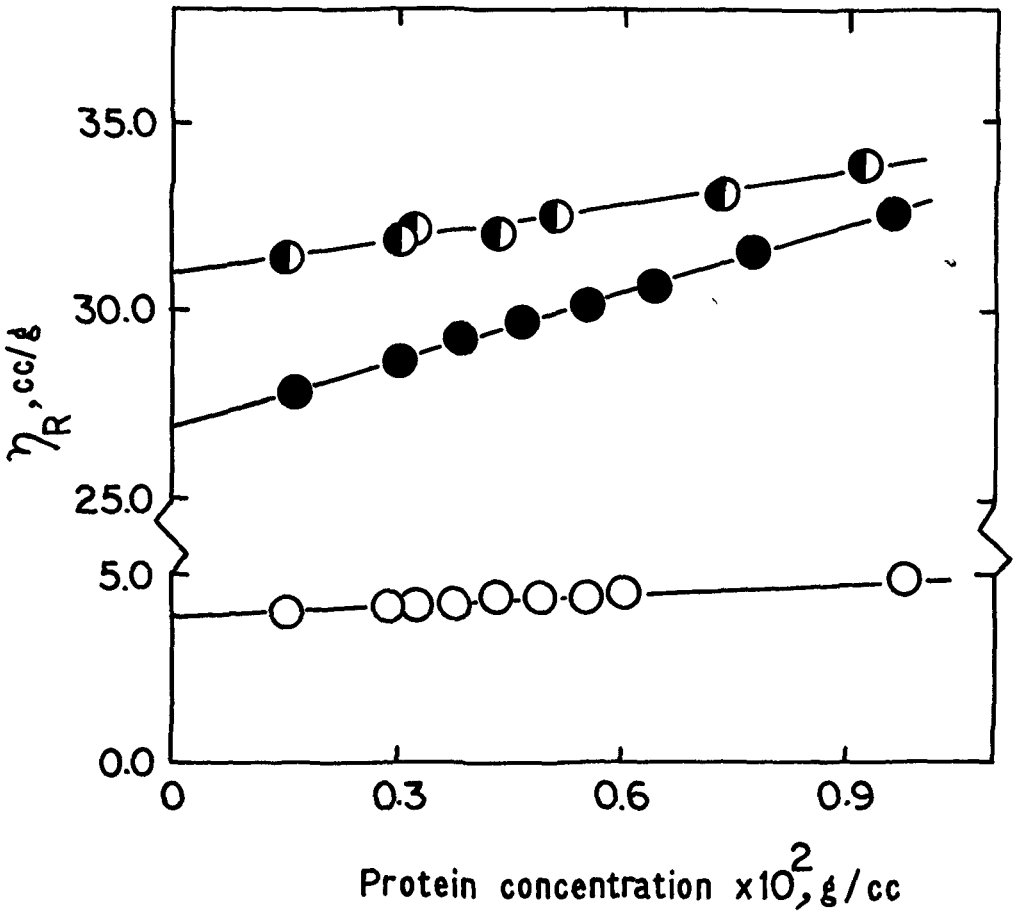

Figure 5. The Intrinsic Viscosity of the Native and the Denatured Oval A_1 at 25°. Experimental conditions: Open circles, protein in phosphate buffer, pH 7.0, ionic strength 0.15; filled circles Oval A_1 in 6 M Gdn.HCl, pH 6.8; and half filled circles, Oval A_1 in 6 M Gdn.HCl plus 0.1 M β-mercaptoethanol, pH 5.1.

gram dry protein, and the summation extends over all solvent components. If we assume that Oval A_1 binds mostly water for which v_1^o = 1 cc/g, we can calculate δ by the help of Equation 3. By setting ν = 2.5, $[\eta]$ = 3.85 cc/g, and $\bar{v}_2$ = 0.748 cc/g (24), the value of δ was computed to be 0.79 which is obviously an overestimation of the extent of hydration. According to Dull and Breese (83), one gram of ovalbumin binds 0.37 gram of water which in the presence of other ions would be less. If the more representative value is 0.2 g/g dry protein as has been suggested elsewhere (24), the value of ν computed by Equation 3 comes out to be 4.1 which corresponds to an axial ratio of 3.5 and to a frictional ratio of 1.15. The latter is in good agreement with 1.17 determined by Lamm and Palson (46).

The ultraviolet absorption spectrum of native Oval A_1 is shown in Figure 4. The specific extinction coefficient of Oval A_1, $E^{1\%}_{1cm}$ at 280 nm (where maximum absorption occurred) in phosphate buffer, pH 7.0, ionic strength 0.20 and at 25° was determined to be 7.150 ± 0.005.

B. The Denatured State of Oval A_1

Tanford and co-workers (66-70) have shown that proteins dissolved in 6 M Gdn.HCl lose all the elements of their native conformation and exist either as cross-linked or linear random coils depending upon whether the protein contains intact or reduced

disulphide bonds. For linear random coil proteins, the molecular weight dependence of the intrinsic viscosity at 35° has been described by the equation (48),

$$[\eta]_{Mo} = 76.1\ n^{0.660} \qquad (4)$$

We have found that this equation also applies to Oval A_1 in 6 M Gdn.HCl plus β-mercaptoethanol. Further, temperature dependence of $[\eta]$ for several proteins is characterized by the decrease in intrinsic viscosity with increase in temperature from 35 to 35° followed invariably by an increase in $[\eta]$ at 40°; further increase in temperature from 40 to 55° produced monotonous decrease in $[\eta]$. This anomaly is shared by all the linear randomly coiled proteins but not by cross-linked random coils, i.e., proteins in 6 M Gdn.HCl alone. As expected, the temperature dependence of $[\eta]$ of Oval A_1 in 6 M Gdn.HCl follows regular decrease with increase in temperature from 15 to 55°. These results suggest that Oval A_1 in 6 M Gdn.HCl probably exists as a cross-linked random coil. The values of intrinsic viscosity of Oval A_1 in 6 M Gdn.HCl and in 6 M Gdn.HCl plus β-mercaptoethanol were found to be 27 cc/g and 31 cc/g, respectively, (see Figure 5) which would imply that cysteine residues forming disulphide bond are proximal and form a very short loop of amino acids.

As stated earlier, the native Oval A_1 has an absorption maximum at 280 nm (see Figure 4), which in 6 M Gdn.HCl appeared as

two maxima at 277 and 281.5 nm (Figure 4). A slight blue shift (1.5 nm) presumably reflects the unfolding of protein. A plot of $\Delta\epsilon_M$ versus wavelength is shown in figure 4, where $\Delta\epsilon_M$ is the difference in the molar extinctions of Oval A_1 under native and denatured conditions. The main features of the difference spectrum were a minimum at 288 nm, a shoulder at 282 nm, and a small minimum at 293 nm. The minimum at 287 nm has been attributed primarily to the perturbation of tyrosine residues of the protein (84). It thus follows that the guanidine hydrochloride induced denaturation of Oval A_1 primarily perturbs the spectrum of its tyrosine residues.

C. Isothermal Denaturation

1. Viscosity Measurements.

The guanidine hydrochloride induced transition of Oval A_1 as measured by the measurements of η_R at pH 7.0 and 25° is shown in Figure 6. It is clear that the experimental points obtained both from denaturation and renaturation studies fall on the same curve which suggests that the Gdn.HCl induced transition of Oval A_1 is thermodynamically reversible. The experimental points in the transition region represent the average of two or three independent measurements which agreed within ± 1%. The transition occurred in the Gdn.HCl concentration range of 1.0 - 4.5 M. The transition appears to be complete at 4.5 M, where η_R was 29.2 cc/g which is identical to the value (29.3 cc/g) found for the protein in

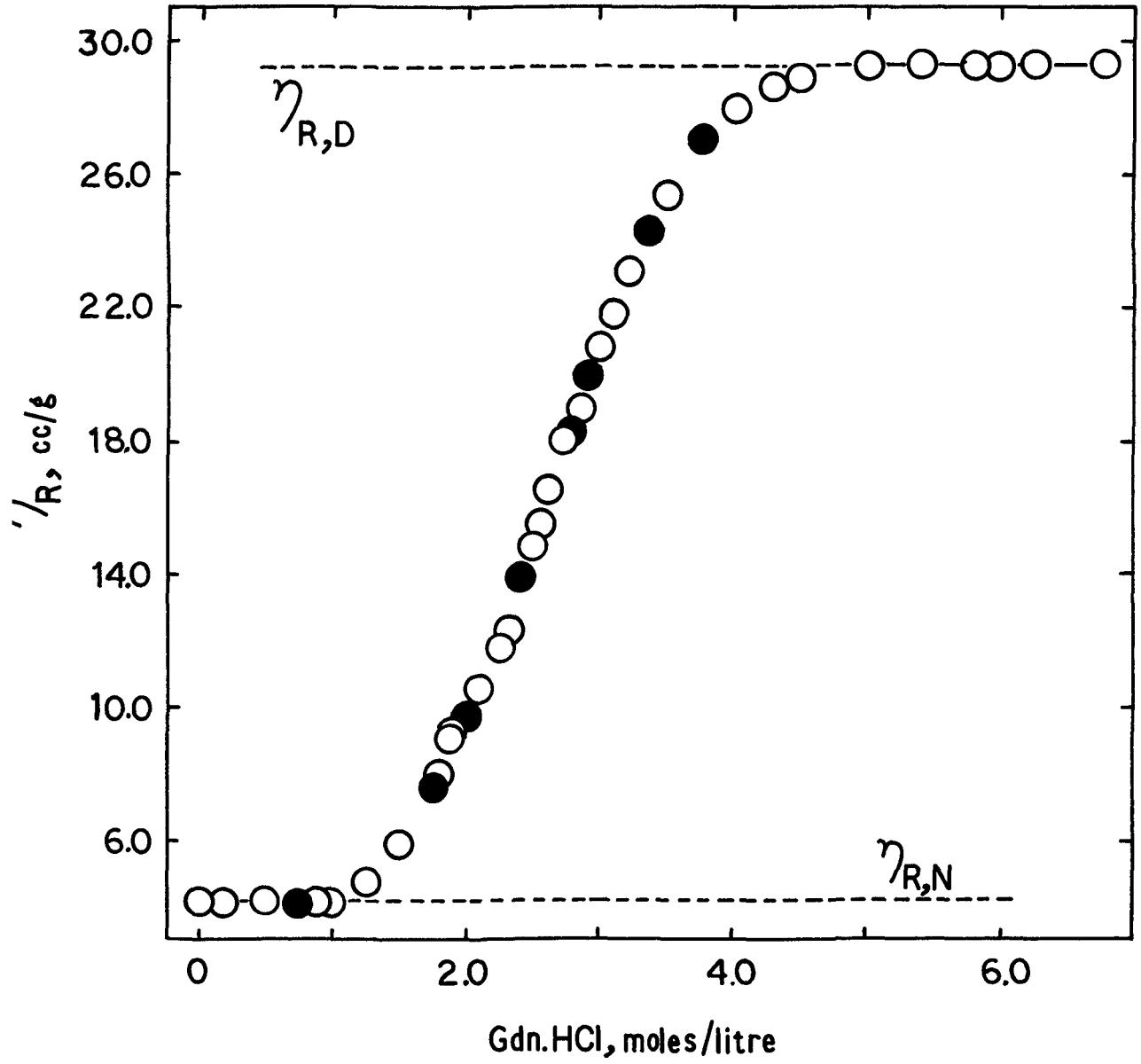

Figure 6. The effect of Gdn.HCl Concentration on the Reduced Viscosity of Oval A_1. Experimental conditions: Protein concentration 0.38%, pH 7.0; open circles represent denaturation experiments and filled circles represent renaturation experiments.

in 6 M Gdn.HCl. The mid point of the transition is near 2.65 M. The value of 29.2 cc/g for $\eta_{R,D}$ was taken to represent the denatured state of Oval A_1 and does not show any detectable dependence on Gdn.HCl concentration. The native state of Oval A_1 has been characterized by a value of 4.24 cc/g for $\eta_{R,N}$.

3. Difference Spectral Measurements.

The guanidine hydrochloride induced transition was also studied by difference spectral measurements under conditions described in Figure 7. Since difference spectrum of the native ovalbumin measured against the protein in 6 M Gdn.HCl showed three distinct maxima at wavelengths 265, 288 and 293 nm, the transition was studied at all the three wavelengths (see Figure 4). In Figure 7, ΔE represents the change in optical density of 1% native protein solution produced by Gdn.HCl denaturation. The transition occurred in the range 1.0 - 4.5 M Gdn.HCl concentration. The guanidine hydrochloride concentrations corresponding to 25, 50 and 75% change are respectively 2.20, 2.65 and 3.2 M in all the three cases (see Figure 7). Further, the change in ΔE, monitored at the three wavelength, with increase in Gdn.HCl concentration seems to correspond the same transition.

The guanidine hydrochloride induced denaturation of Oval A_1 is reversible because the points obtained from the denaturation and renaturation experiments seem to fall on the same curve (see Figure 7). Unlike reduced viscosity, the value of ΔE for the

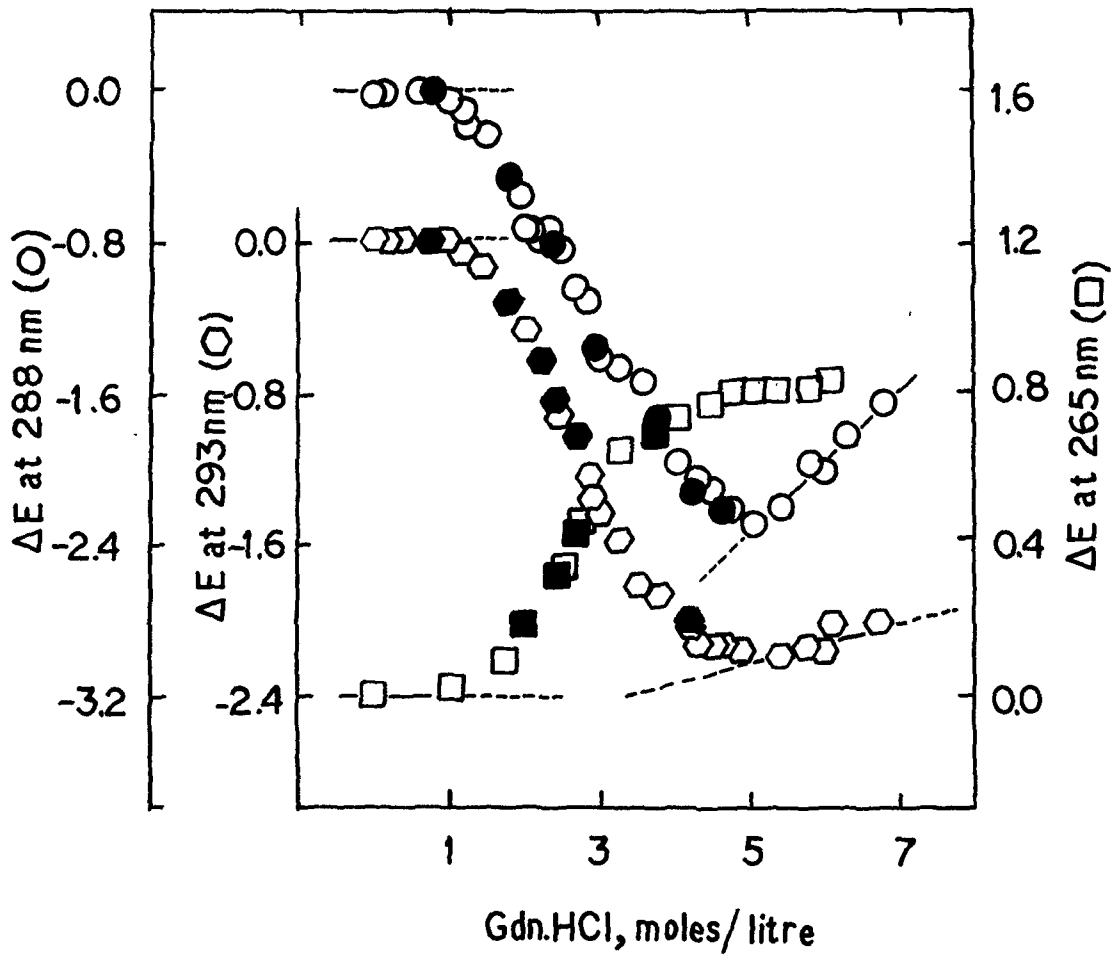

Figure 7. Effect of Gdn.HCl Concentration on the Difference Absorption of Oval A_1 at 265, 288 and 293 nm. Experimental conditions: Temperature 25°; pH 7.0; open squares, circles and hexagons represent results from denaturation experiments while the filled ones represent results from renaturation experiments. The reference solution was Oval A_1 in phosphate buffer, pH 7.0, ionic strength 0.15.

denatured protein, ΔE_D, showed dependence on Gdn.HCl concentration, C_g, which can be described by the expressions

$$(\Delta E_D)_{288} = 0.369\ C_g - 4.163 \qquad (5)$$

$$(\Delta E_D)_{293} = 0.093\ C_g - 2.630 \qquad (6)$$

The values of ΔE for the native protein, ΔE_N, does not seem to be dependent on Gdn.HCl concentration. It should be noted that the total change in ΔE caused by increasing Gdn.HCl concentration was 15% at 283 nm, 51% at 288 nm and 71% at 293 nm.

3. Analysis of the Guanidine Hydrochloride Induced Transition.

It will be assumed that the Gdn.HCl induced transition of Oval A_1 involves one step and that the intermediate states, if any, are too unstable to make any detectable contribution to the properties used in the study of the transition. The assumption of a two-state transition appears to be supported by the evidence given below:

(i) The per cent denaturation measured by two independent properties is same throughout the transition (see Figure 8). The reduced viscosity provides a measure of the gross conformation of the protein, whereas, difference spectral measurements would indicate the changes in the environments of the chromophores in

Figure 8

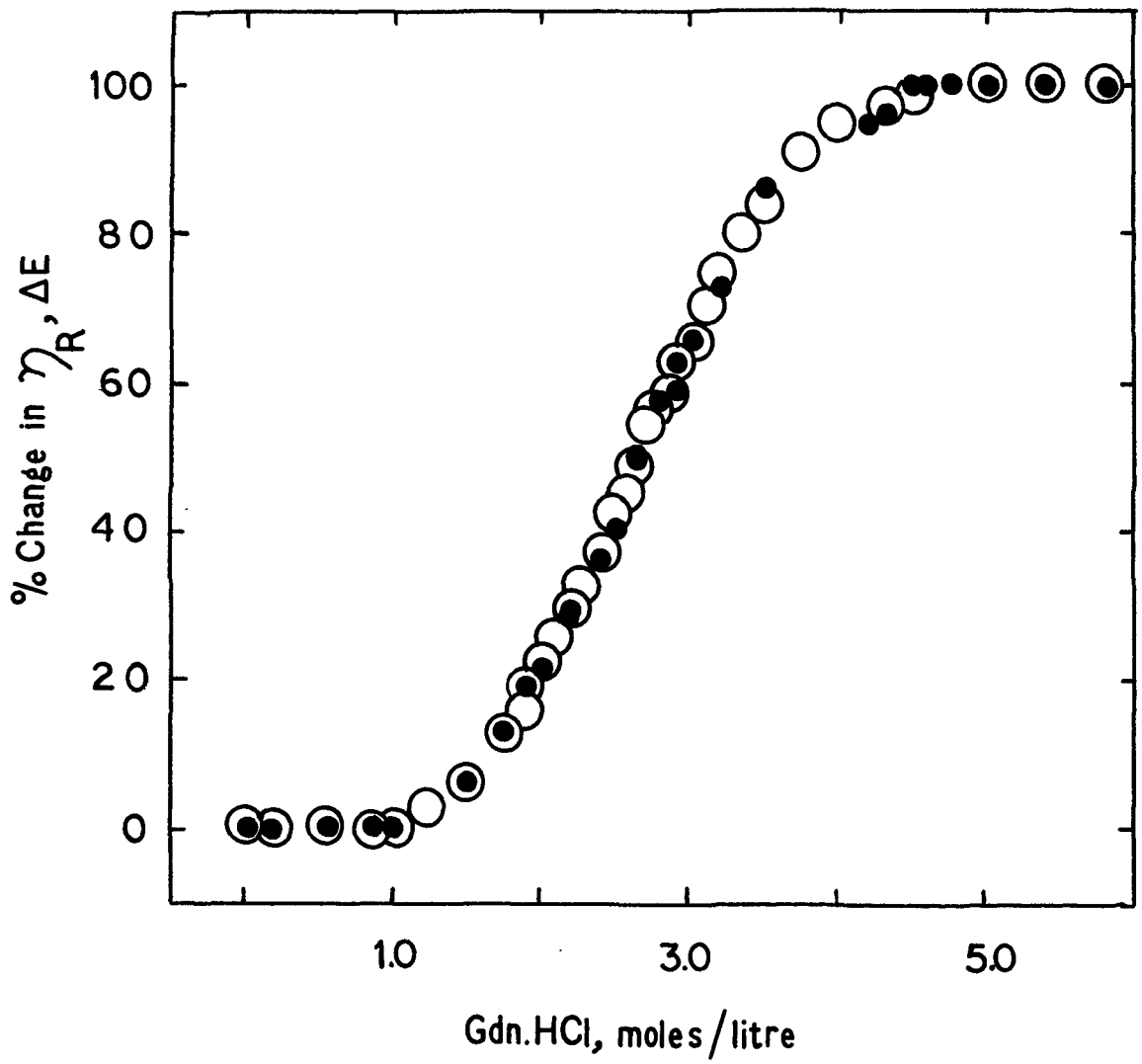

Figure 8. Per Cent Change in Reduced Viscosity and Difference Spectral Intensity as a Function of Gdn.HCl Concentration. Open circles represent data calculated from viscosity measurements (Figure 6) and filled circles represent results calculated from difference spectral measurements (Figure 7).

the protein. If both measures the same transition as they do in the present case, the transition is most likely to be a one-step process. However, this is a necessary but not a sufficient condition of a two-state transition.

(ii) More direct conclusion can be reached by the kinetic results given in Figure 9. The process of denaturation and renaturation followed first order kinetics. Further, the value of η_R extrapolated to infinity agreed with those found in equilibrium studies. The guanidine hydrochloride denaturation as well as renaturation of Oval A_1 followed first order kinetics under variety of conditions, namely, Gdn.HCl concentration (1.8 to 3.1), pH (2.0 to 7.0) and temperature (15 to 45^0).

4. Dependence of Equilibrium Constant on the Activity of Gdn.HCl.

The denaturation of Oval A_1 by Gdn.HCl may be expressed as

$$N \rightleftharpoons D, \qquad (7)$$

where N and D represent the native and denatured states. The equilibrium constant, K, for the reaction 7 can be expressed in terms of the measured properties η_R and ΔE by the help of the following equations,

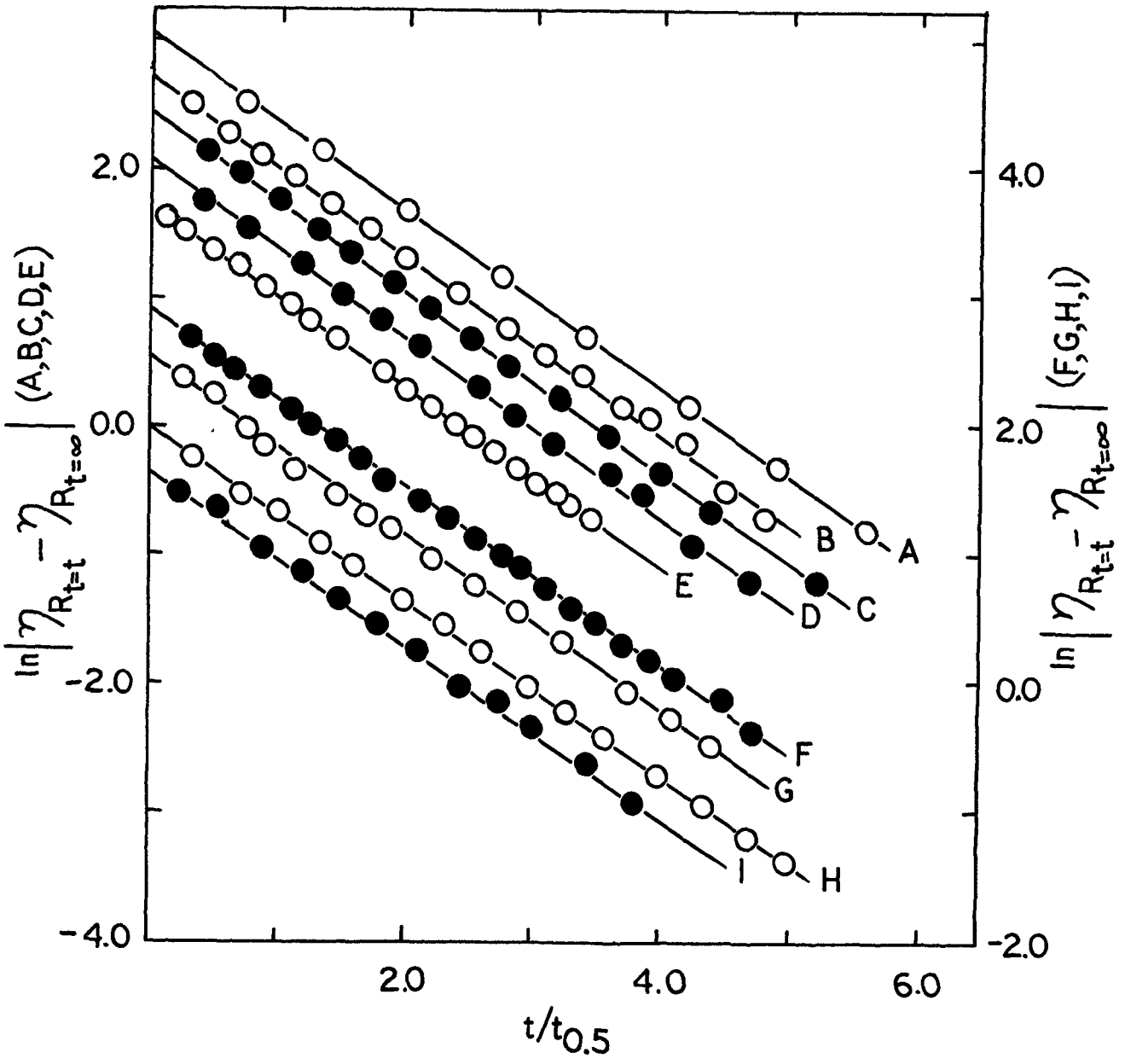

Figure 9. First Order Kinetic Plot for Denaturation and Renaturation of Oval A_1 at Different Gdn.HCl Concentrations, pH's and Temperatures. Experimental conditions: Open circles represent results from denaturation experiments at 2.0 M Gdn.HCl, pH 7.0, 45° (A); 1.72 M Gdn.HCl, pH 3.0 25° (B); 2.0 M Gdn.HCl, pH 7.0, 25° (E); 2.20 M Gdn.HCl, pH 5.2, 25° (G); and 2.30 M Gdn.HCl, pH 7.0, 15° (H). Filled circles represent results from renaturation experiments at 1.25 M Gdn.HCl, pH 2.2, 25° (C); 1.90 M Gdn.HCl, pH 3.8, 25° (D); 2.30 M Gdn.HCl, pH 6.6, 25° (F); and 2.24 M Gdn.HCl, pH 4.8, 25° (I).

$$K = \frac{\eta_R - \eta_{R,N}}{\eta_{R,D} - \eta_R} \qquad (8)$$

$$K = \frac{\Delta E - \Delta E_N}{\Delta E_D - \Delta E} \qquad (9)$$

In Equations 8 and 9, η_R and ΔE represent the observed reduced viscosity and difference spectral intensity, respectively.

The dependence of equilibrium constant on the concentration or activity of Gdn.HCl is evident from Figure 10. The values of K were calculated from the data of Figures 6 and 7 in the range $0.3 \leq K \leq 5$. The activity of Gdn.HCl, a_g, was calculated using the equation (85),

$$\log a_g = -0.51907 + 1.48386 \log C_g - 0.25618 (\log C_g)^2 + 0.58844 (\log C_g)^3 \qquad (10)$$

The relation between K and a_g has been described by Tanford (86),

$$K = K^0 \frac{\prod_{i=1}^{n_D} (1 + k_{i,D}\, a_g)}{\prod_{i=1}^{n_N} (1 + k_{i,N}\, a_g)}, \qquad (11)$$

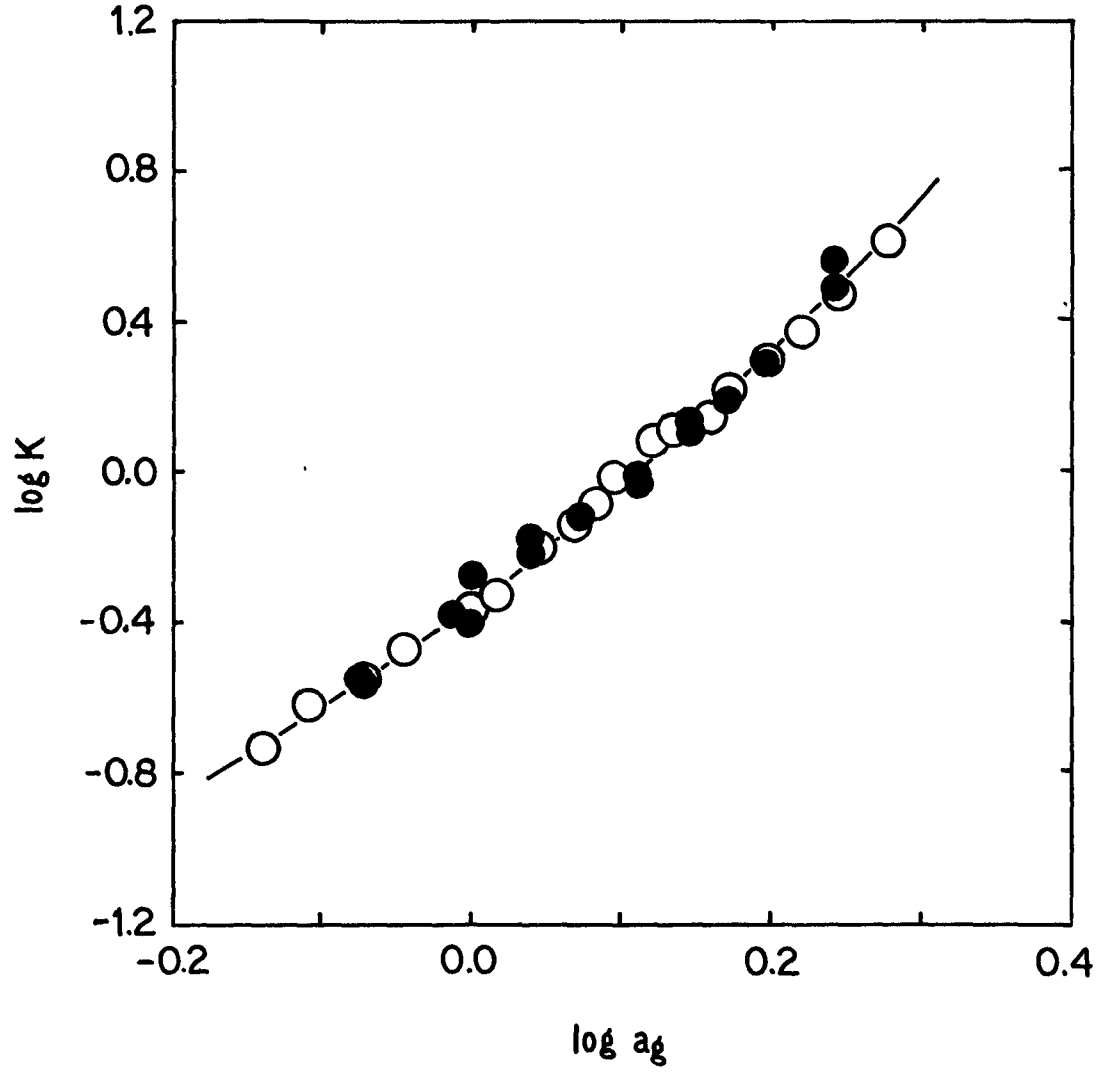

Figure 10. Effect of Gdn.HCl Activity on the Logarithm of Equilibrium Constant for the Transition of Oval A_1 at 25°, pH 7.0. Experimental conditions: Open circles represent data calculated from viscosity measurements (Figure 6), filled circles represent data from difference spectral measurements (Figure 7), solid line was drawn according to Equation 12. The experimental points represent the values of K in the range $0.2 \lesssim K \lesssim 5.0$.

where K^o is the equilibrium constant in absence of the denaturant, n_N and n_D represent the number of moles of Gdn.HCl that may bound per mole of the native and denatured protein, respectively, and $k_{i,N}$ is the intrinsic binding constant at each individual site on the native protein, and $k_{i,D}$ a similar parameter for the denatured protein. If all the sites on the native and denatured molecules are identical and non-interacting, $k_{i,N}$ and $k_{i,D}$ may be replaced by a single constant k. Equation 11 will then reduce to

$$K = K^o (1 + k\, a_g)^{\Delta n} \qquad (12)$$

where $\Delta n = n_D - n_N$. The term $(1 + k\, a_g)^{\Delta n}$ of Equation 12 describes the dependence of K on the activity of Gdn.HCl and is assumed to be independent of pH and temperature. We may denote it by another equilibrium constant, K(G),

$$K(G) = (1 + k\, a_g)^{\Delta n} \qquad (13)$$

Here the assumption is that $Gdn.H^+$ ion is much more effective than Cl^- ion so that the effect of Cl^- ion is unimportant. In the absence of relevant data on the activity of $Gdn.H^+$ ions in the existing literature, we will use mean ion activity, $a_\pm$, which is $a_g^{1/2}$. Thus, Equations 12 and 13 becomes

$$K = K^{o} (1 + k a_{\pm})^{\Delta n} \qquad (14)$$

$$K(G) = (1 + k a_{\pm})^{\Delta n} \qquad (15)$$

5. Analysis of the Equilibrium Data.

The results of Figure 6 and 7 are analyzed according to Equation 12 by assuming that bound ligand is a neutral Gdn.HCl molecule. The results of such analysis are shown in Figure 10, where open circles represent the viscosity results and filled circles represent experimental points obtained from difference spectral measurements. The best values of K^{o}, k and Δ n were obtained by least squares method using a computer programme written in Fortran. At 25° the values of K^{o}, k and Δ n were, respectively 8.746×10^{-3}, 0.806 and 6.680. By similar procedure, the value of K^{o}, k and Δ n were determined using Equation 14 and were found to be 4.53×10^{-5}, 1.115 and 12.280, respectively. The free energy change in absence of Gdn.HCl was 6 Kcal/mole.

D. Effect of pH on Denaturation

The reduced viscosity of native Oval A_1 in 0.15 M NaCl, $\eta_{R,N}$, was measured at different pH values and the results are shown in Figure 11. Evidently, $\eta_{R,N}$ is independent of pH up to 3.5, below which η_R increased presumably due to acid unfolding of Oval A_1.

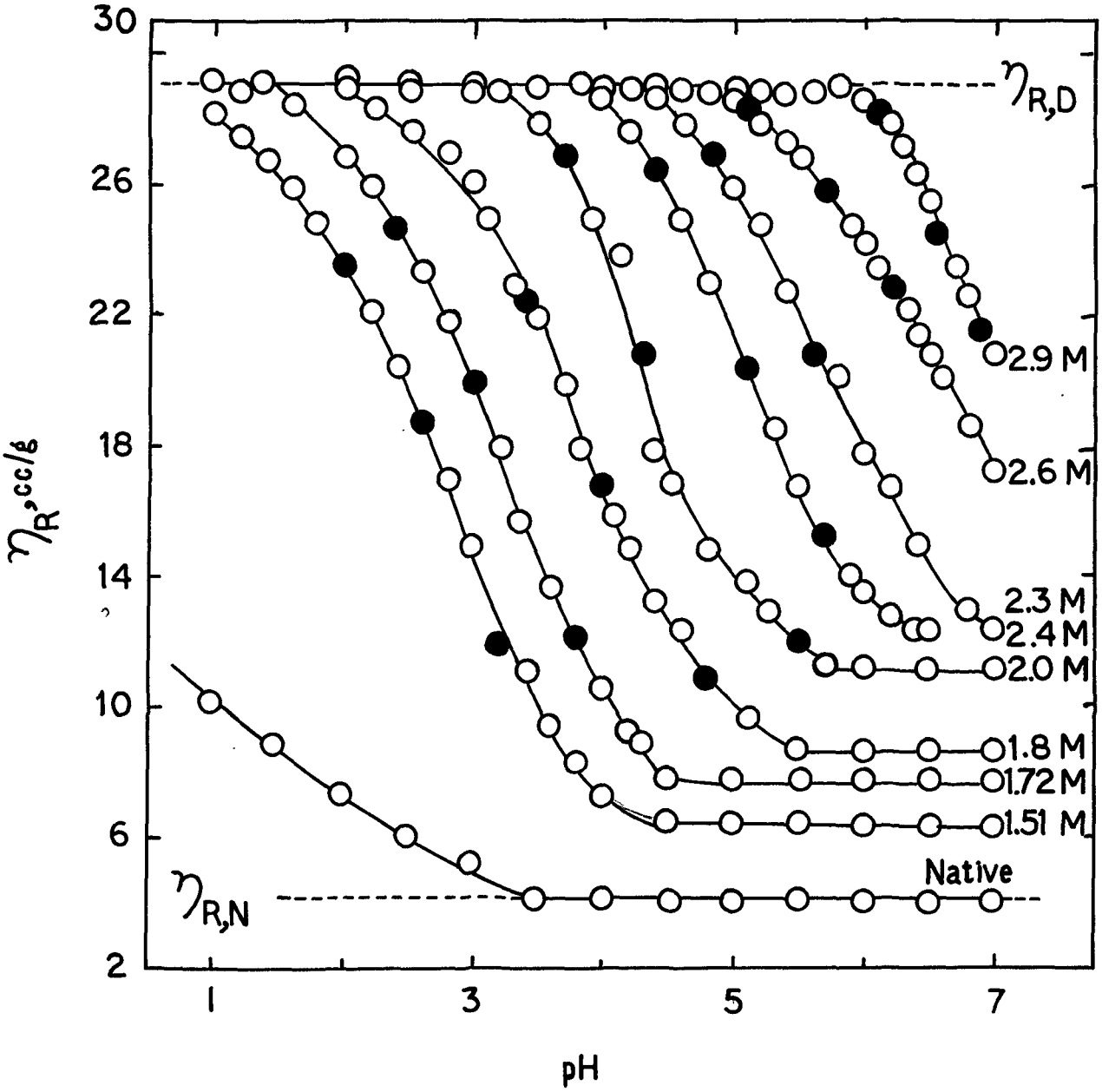

Figure 11. Effect of pH on the Gdn.HCl Denaturation of Oval A_1 at 25°. Experimental conditions: The filled circles were obtained by bringing the pH to the desired value after exposure of the protein solution to pH 1.0. The Gdn.HCl concentration for each curve is shown on the curve.

The value of $\eta_{R,N}$ was measured to be 4.24 cc/g in 0.15 M NaCl at pH 7.0 and 25°. From the limiting values of η_R at various Gdn.HCl concentrations, it was found that $\eta_{R,D}$ is independent of pH and Gdn.HCl concentration (see Figure 11). Incidentally, the value of $\eta_{R,D}$ was same (i.e., 29.2 cc/g) as that obtained for cross-linked randomly coiled Oval A_1 in 6 M Gdn.HCl at 25°.

The pH induced Gdn.HCl denaturation of Oval A_1 was studied at different concentrations of Gdn.HCl in the range 1.51 - 2.90 M, and the results are shown in Figure 11: the results at two Gdn.HCl concentrations, i.e., 1.90 and 2.12 M have been omitted for clarity. It is clear that the decrease in pH from 7.0, favoured significant increase in η_R at all Gdn.HCl concentrations. As expected, the height of the pH induced transition decreases with increase in Gdn.HCl concentration. The transitions shown in Figure 11 were found to be reversible; the value of η_R of the protein solution which was previously exposed to pH 1.0 fall on the same curve drawn through the experimental points obtained for denaturation. Further, the kinetic experiments, described earlier (see page 43) suggested that the pH induced Gdn.HCl denaturation of Oval A_1 is a two-state process. Thus, there is some justification in analysing the data of Figure 11 assuming a two-state process.

Since the equilibrium constant, K, for the process $N \rightleftharpoons D$, could be written as a product of three independent functions, i.e.,

$$K = K^0 F(a_H) K(G), \qquad (16)$$

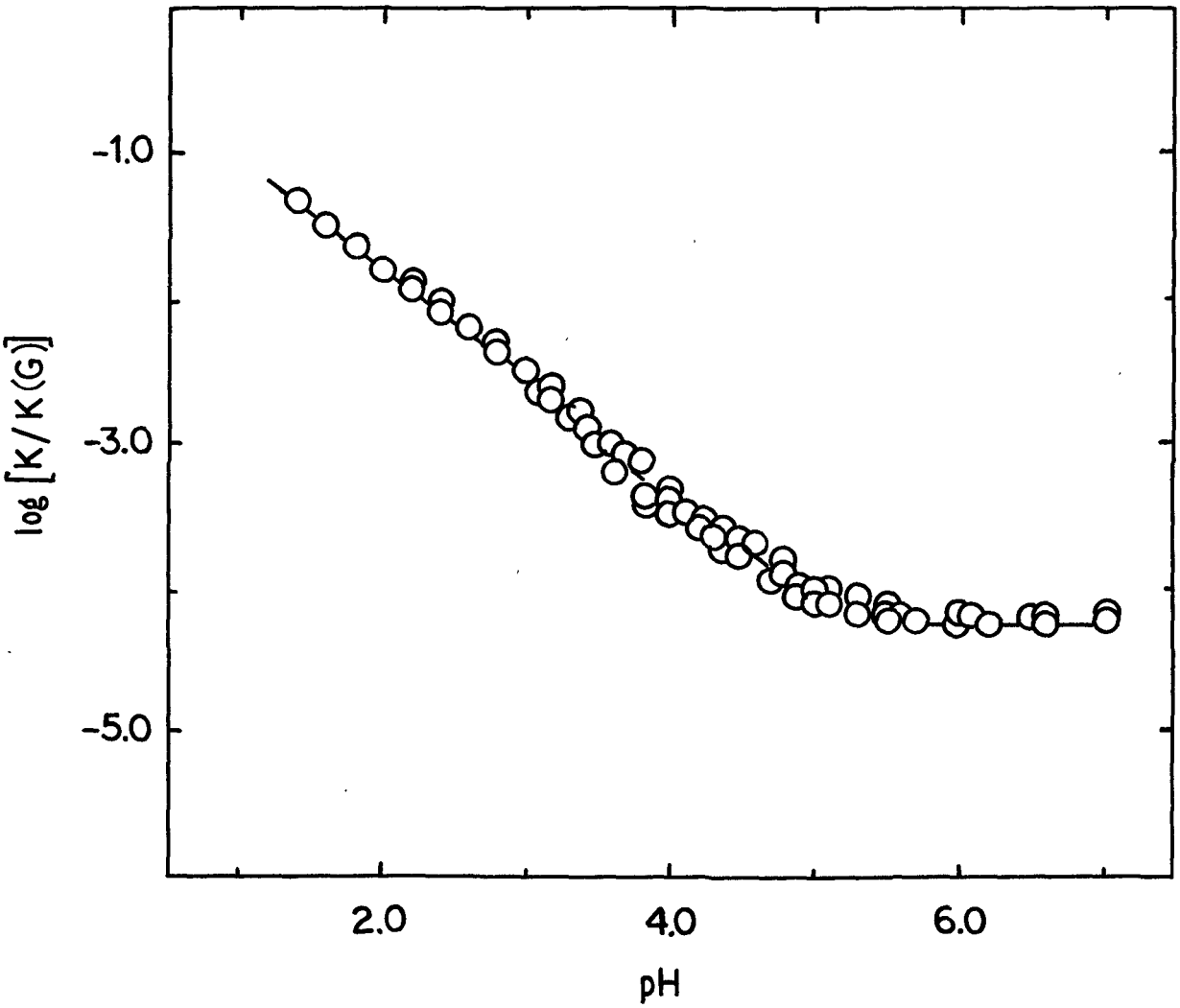

Figure 12. Effect of pH on the Logarithm of the Equilibrium Constant for the Gdn.HCl Denaturation of Oval A_1. The values of equilibrium constants were calculated from the data of Figure 11, and were plotted as log[K/K(G)] versus pH, with K(G) given by Equation 13. The experimental points represent the values of K in the range $0.2 \leq K \leq 5.0$.

where $F(a_H)$ describes the dependence of K on pH, and K^0 and K(G) have the same meaning as in Equations 12 and 13. The values of K(G) have been determined earlier (page 48). Thus, the term K/K(G) will be independent of Gdn.HCl concentration. A plot of log [K/K(G)] versus pH is shown in Figure 12. It can be seen that results obtained at all Gdn.HCl concentrations can be described by a single curve. This would mean that the function K(G) determined at pH 7.0 applies at lower pH also, and thus indicates the mutual independence of K(G) and $F(a_H)$.

The maximum value of -dlogK/dpH up to pH 5.0 is about 1, which means that one titrable group must have different pK values in the native and denatured states. By all indications this group appears to be a carboxyl one. This group could not be unequivocally determined by the procedure of Aune et al. (85).

E. Effect of Temperature on Denaturation

1. Native State.

The state of Oval A_1 in phosphate buffer, pH 7.0, ionic strength 0.15, represents the native state of the protein. Figure 13 shows the effect of temperature on η_R of the native Oval A_1. It is evident from the curve B (Figure 13), that the value of $\eta_{R,N}$ does not show any detectable temperature dependence up to 50° beyond which η_R increases with increase in temperature. The change in η_R above 50° is not a change in the value of $\eta_{R,N}$,

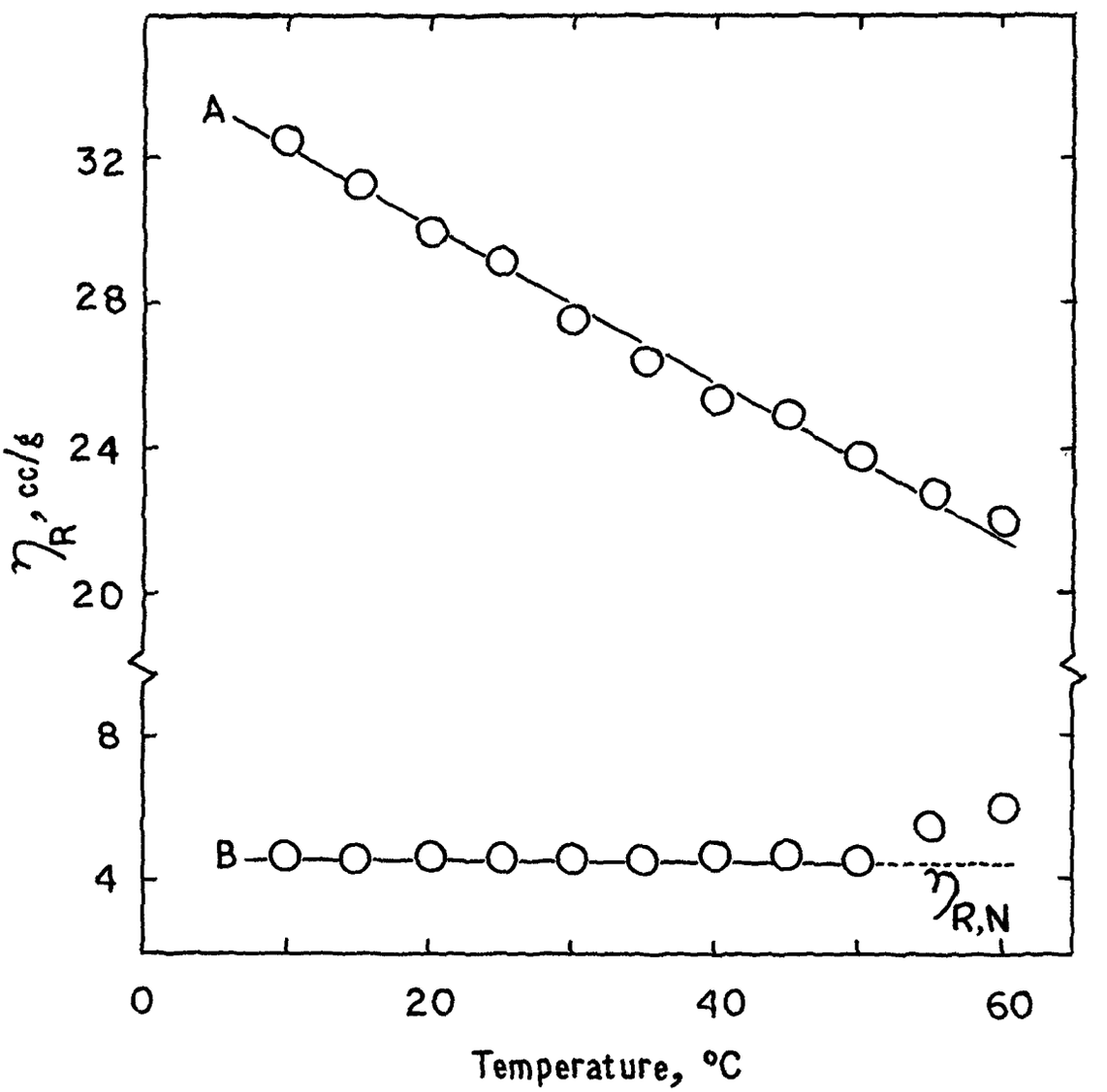

Figure 13. Temperature Dependence of the Reduced Viscosity of the Native and the Denatured Oval A_1. Experimental conditions: Protein concentration 0.38%, curve A shows the results for the protein in 6 M Gdn.HCl., pH 7.0; and curve B represents the data for the native protein in phosphate buffer, pH 7.0, ionic strength 0.15.

but presumably reflects the disruption of the native conformation caused by thermal denaturation (87,89). Therefore, in the calculation of the equilibrium constant at different temperatures, the value of $\eta_{R,N}$ at 25° has been used.

2. Denatured State.

The upper curve of Figure 13 shows the values of η_R of Oval A_1 in 6 M Gdn.HCl as a function of temperature. The reduced viscosity of the denatured protein, unlike the native one, decreased monotonically with an increase in temperature. The decrease in η_R for proteins in 6 M Gdn.HCl, where they behave as cross-linked random coils, seems to be the characteristics of the denatured proteins with disulphide bonds intact (48). The values of $\eta_{R,D}$ were found to be independent of Gdn.HCl concentration at all temperatures. The temperature dependence of $\eta_{R,D}$, as shown by the curve A of Figure 13, is described by the following relation obtained by the help of least squares,

$$\eta_{R,D} = 0.209t' + 34.21, \qquad (17)$$

where t' is the temperature in degree centigrade. The value of $\eta_{R,D}$ at a given temperature was computed from Equation 17.

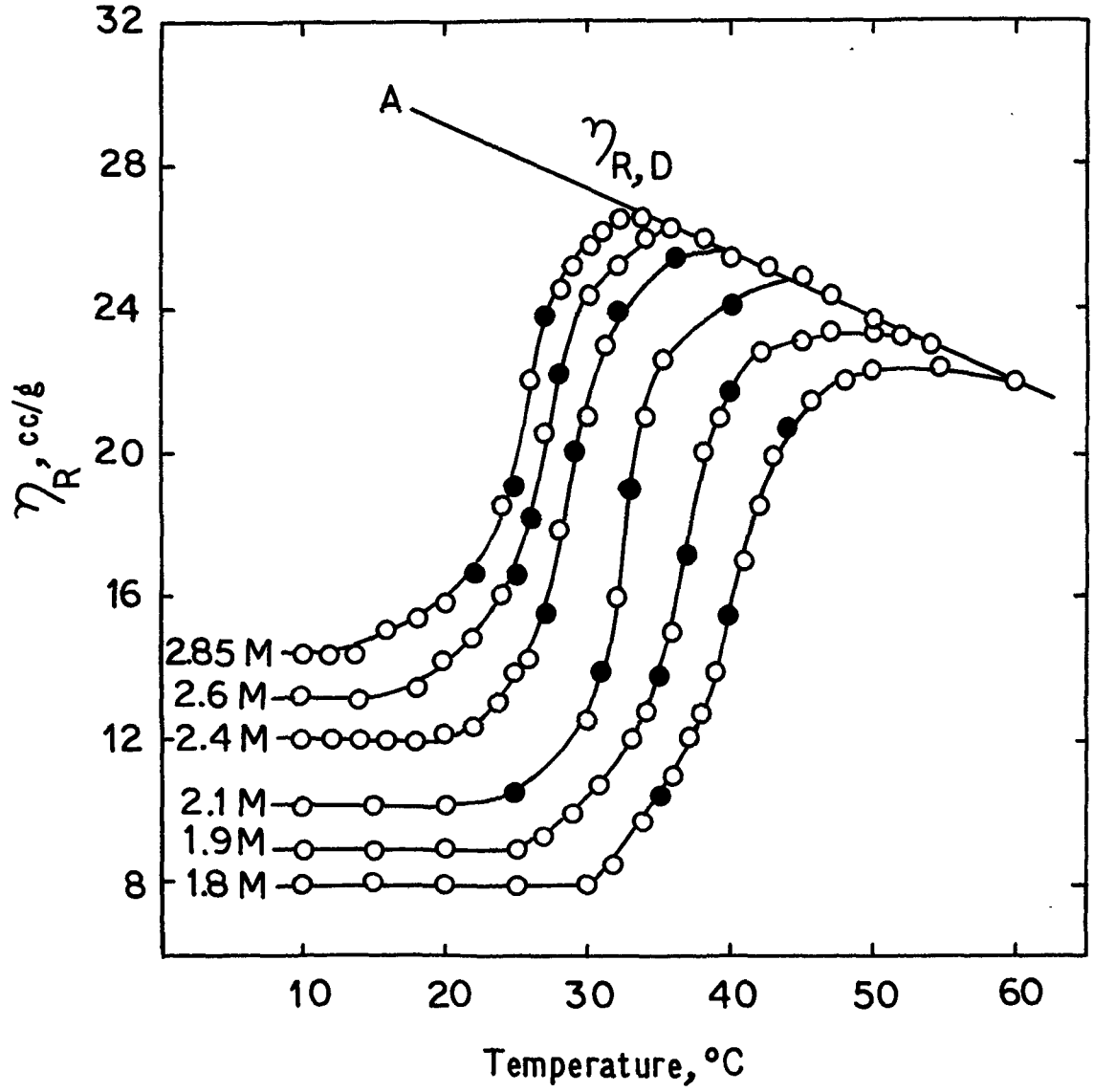

Figure 14. Effect of Temperature on the Gdn.HCl Denaturation of Oval A_1. Experimental conditions: Protein concentration 0.38%. The filled circles represent data obtained at a given temperature both by heating and cooling the protein solution. Curve A was drawn according to Equation 17.

3. Effect of Temperature on Equilibrium Constant.

The effect of temperature on the Gdn.HCl induced denaturation of Oval A_1 at various concentrations of the denaturant, was investigated in the temperature range 10 - 60°. Figure 14 shows a plot of η_R versus temperature for representative data, where it can be seen that an increase in temperature favours denaturation. In order to maintain clarity some points in the post-transition part were omitted. The criterion of the reversibility of the thermal denaturation was to see if the same value of η_R was obtained at a given temperature both by heating and cooling the protein solution containing Gdn.HCl. The values of η_R thus obtained are shown in Figure 14. It can be seen in Figure 14 that filled and open circles lie on the same curve. This suggested that the temperature induced Gdn.HCl denaturation of Oval A_1 is a reversible process.

The plot of lnK versus 1/T given in Figure 15 showed curvature suggesting the involvement of the term for heat capacity change at constant pressure. The latter is the characteristics of protein denaturation. The solid lines in Figure 15 were drawn by the method of least squares using the experimental points. The data are seen to fit the following equation,

$$\ln K = A + B/T + CT + DT^2, \qquad (18)$$

where A,B,C and D are temperature independent constants. As can be

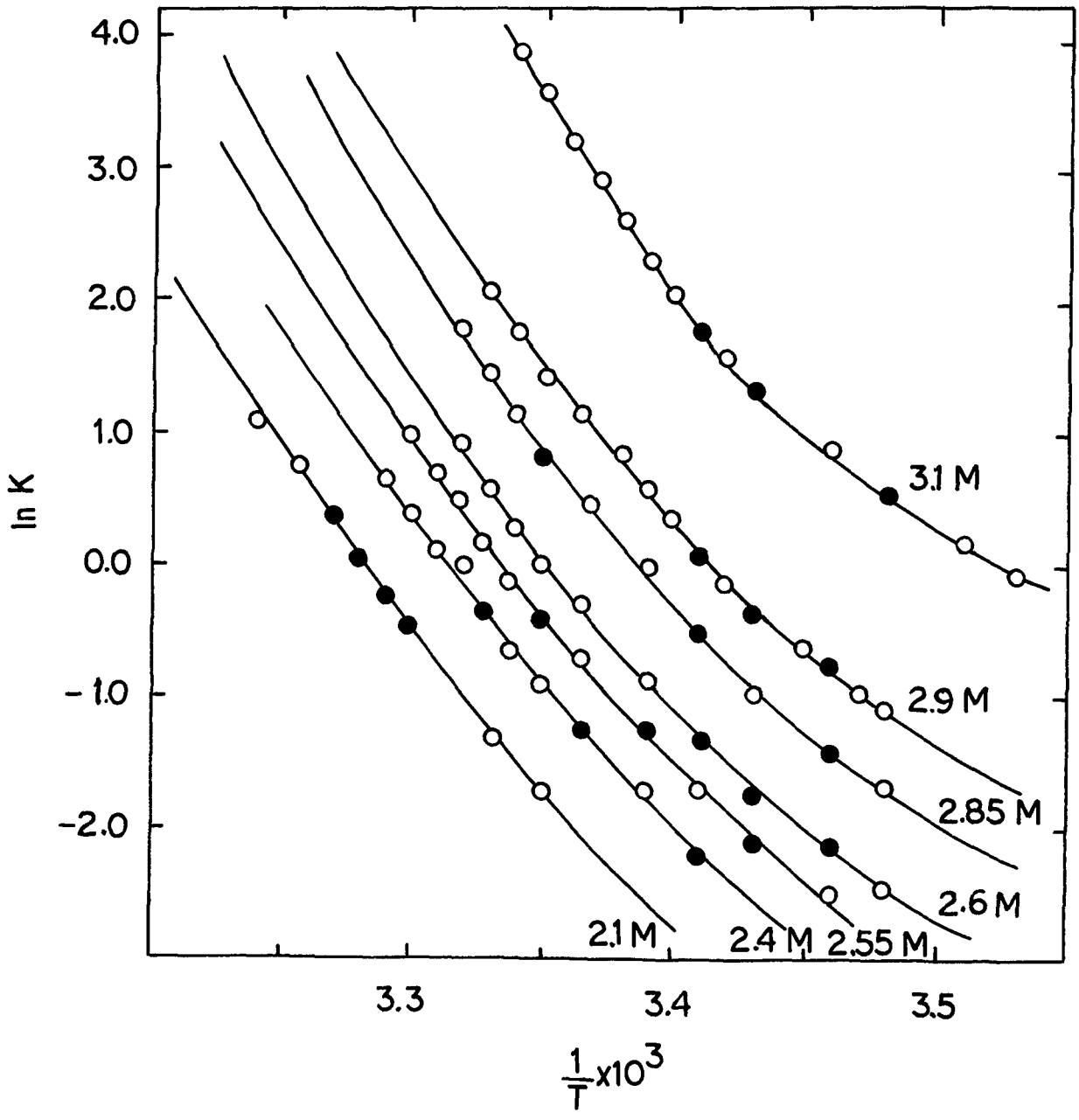

Figure 15. Temperature Dependence of the Equilibrium Constant for the Gdn.HCl Denaturation of Oval A_1. Experimental conditions were the same as given in Figure 14. The open and filled circles have the same significance.

seen in Figure 15, the observed temperature dependence of equilibrium constant at different Gdn.HCl concentration is accurately described by Equation 18. The values of the coefficients are listed in Table III and seem to be independent of Gdn.HCl concentration.

4. Change in Enthalpy, Entropy and Free Energy for Denaturation.

From the temperature dependence of K, the change in enthalpy, ΔH, for the Gdn.HCl induced transition of Oval A_1 was calculated by the help of Equations 19 and 20,

$$\left(\frac{\partial \ln K}{\partial 1/T}\right)_p = -\frac{\Delta H}{R}, \qquad (19)$$

$$\Delta H = R\left[-B + CT^2 + DT^3\right], \qquad (20)$$

where R is gas constant equal to 1.9872 cal $mole^{-1}$ deg^{-1}, and T is temperature in ^{o}K. Since denaturation does not involve any change in the number of protein molecules, apart from the effect of non-ideality, the thermodynamic parameters ΔH, etc., are independent of concentration unit. Likewise, the entropy change, ΔS, for denaturation is given by the following equation,

$$\Delta S = R\left[A + 2CT + 3DT^2\right] \qquad (21)$$

TABLE III

COEFFICIENTS OF EQUATION 18 AS A FUNCTION OF Gdn.HCl CONCENTRATION

Gdn.HCl concentration (M/L)	$A \times 10^{-3}$	$-B \times 10^{-4}$	C	$D \times 10^{3}$
3.10	1.12399	6.08499	5.78440	9.08925
2.90	1.12399	6.08499	5.78562	9.07327
2.85	1.12399	6.08498	5.78375	9.06000
2.70	1.12400	6.08498	5.78435	9.06370
2.60	1.12398	6.08499	5.78494	9.05445
2.55	1.12400	6.08498	5.78435	9.04782
2.50	1.12400	6.08498	5.78435	9.04620
2.40	1.12400	6.08499	5.78435	9.04206
2.10	1.12400	6.08499	5.78432	9.02863
1.90	1.12400	6.08498	5.78435	9.00832
1.80	1.12400	6.08500	5.78432	8.98695

The free energy change, ΔG, which is $-RT\ln K$, is expressed by the relation,

$$\Delta G = R\left[AT + B + CT + DT^2\right] \qquad (22)$$

The temperature dependence of the thermodynamic parameters ΔH, ΔS and ΔG for Gdn.HCl transition of Oval A_1 is depicted in Figure 16. The curves were computed by Equations 20, 21 and 22, using values of the coefficients given in Table III. With decrease in temperature the values of ΔH and ΔS decreased. At lower temperature, the curvature in the plot of ΔG *versus* T leads to an inversion in the temperature dependence of ΔG. At the point of inversion the following relation is obtained,

$$\left(\frac{-\partial \Delta G}{\partial T}\right)_P = \Delta S = 0 \qquad (23)$$

The value of ΔS is zero near 1^0 which represents the temperature of maximum stability, T_{max}.

5. *The Heat Capacity Change.*

The value of the change in heat capacity, Cp, can be calculated by the help of the equation,

$$\left(\frac{\partial \Delta H}{\partial T}\right)_P = \Delta Cp. \qquad (24)$$

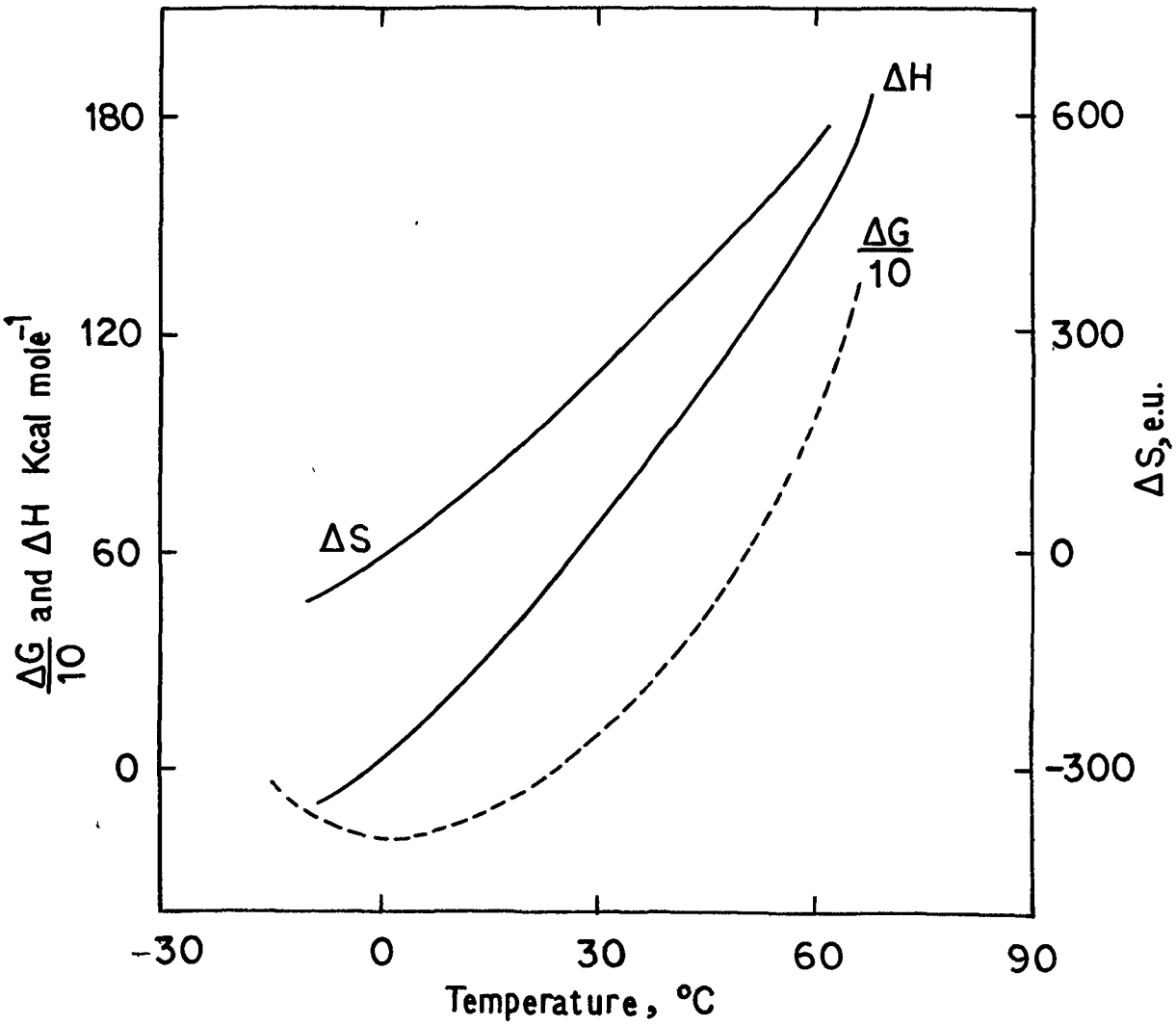

Figure 16. The Temperature dependence of ΔH, ΔS and ΔG for Gdn.HCl Denaturation of Oval A_1. The solid curves for ΔH and ΔS were drawn according to Equations 20 and 21, respectively. The broken curve for ΔG was drawn according to Equation 22.

Thus, from Equation 22 it follows that,

$$\Delta Cp = R \left[2CT + 6DT^2 \right] \qquad (25)$$

Like the thermal denaturation of chymotrypsinogen and ribonuclease (88,89) Δ Cp does not seem to be independent of temperature. The temperature dependence may very well be ascribed to the uncertainty involved in the extrapolation of the data. Further, the calculated value of Δ Cp is very sensitive to the experimental uncertainty. We have, therefore, calculated the average value of Δ Cp in the temperature range 15 - 35°, and the value of Δ Cp comes out to be 3,700 cal $mole^{-1}$ deg^{-1}.

F. Kinetics of Denaturation and Renaturation

The results on the kinetics of denaturation and renaturation obeyed first order kinetics under various conditions of Gdn.HCl concentration, pH and temperature. The representative data are shown in Figure 9. The slope of such a plot yielded an apparent first order rate constant, k_{app}. A plot of log k_{app} *versus* log C_g is shown in Figure 17, where open and filled circles represent results from denaturation and renaturation experiments, respectively. It is evident from Figure 17 that open and filled circles fall on the same smooth curve. This suggests that the same value of k_{app} at a given denaturant concentration will be obtained regardless of the direction of approach to equilibrium.

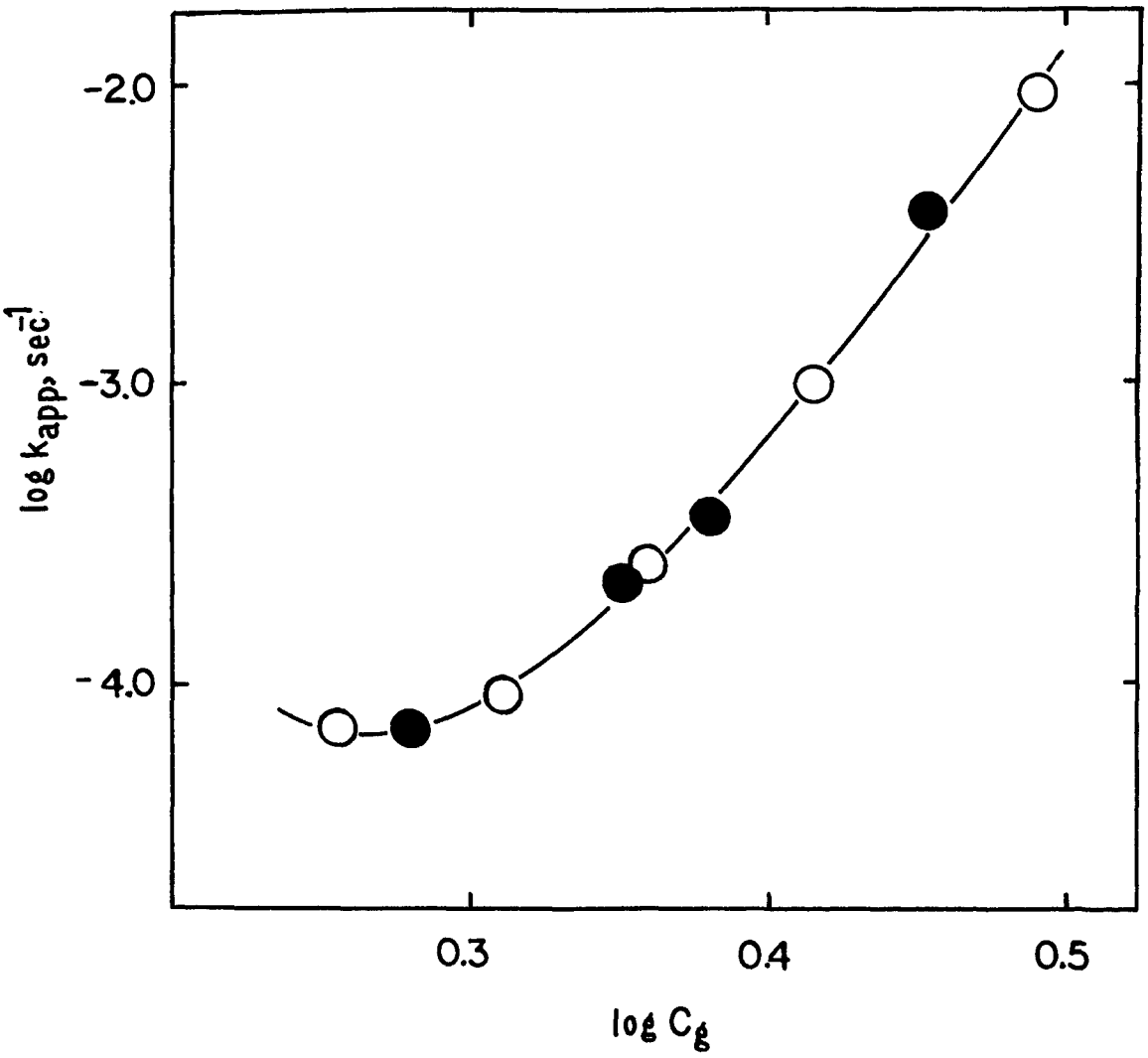

Figure 17. The Dependence of the Apparent Rate Constant on Gdn.HCl Concentration. Experimental conditions: Temperature 25°, pH 7.0, open and filled circles represent results from denaturation and renaturation experiments, respectively.

IV. DISCUSSION

A. Isothermal Denaturation

The intrinsic viscosity of the native Oval A_1 was measured to be 3.9 cc/g which is comparable to the reported values (4.0 - 4.3 cc/g) for the whole ovalbumin. Further, the intrinsic viscosity of Oval A_1 lies in the range 3 - 4 cc/g expected for globular proteins (58). The viscosity data, therefore, suggest that the hydrodynamic volume of Oval A_1 is identical to that of ovalbumin, and that the protein molecule is compact and globular.

The intrinsic viscosities of Oval A_1 with disulphide bond intact and reduced in 6 M Gdn.HCl were determined at 25° and were 27 and 31 cc/g, respectively. The intrinsic viscosity of reduced Oval A_1 in 6 M Gdn.HCl was the same as can be predicted by empirical equations (48,58) describing the molecular weight dependence of intrinsic viscosities of randomly coiled proteins in 6 M Gdn.HCl solution. It, therefore, follows that Oval A_1 behaves as a linear random coil in 6 M Gdn.HCl plus β-mercaptoethanol, and that the hydrodynamic volumes of reduced and unreduced Oval A_1 in 6 M Gdn.HCl are not very different. The latter situation would arise if the disulphide bond involves the proximal cysteine residues. The

difference spectral result suggests an extensive perturbation of the spectra of aromatic amino acid residues particularly phenylalanine and tyrosine residues, produced by the Gdn.HCl induced unfolding.

The denaturation experiments performed by reduced viscosity and difference spectral measurements suggested that the Gdn.HCl induced transition of Oval A_1 is complete at 4.5 M Gdn.HCl. As expected, the reduced viscosity at 4.5 M Gdn.HCl corresponds to an intrinsic viscosity of 27 cc/g which is the value observed in 6 M Gdn.HCl. It is of interest to recall the previous studies of Schellman et al. (72) and Holt and Creeth (44) who found that ovalbumin was completely denatured by Gdn.HCl concentration beyond 4 M.

1. Dependence of the Equilibrium Constant on Gdn.HCl Activity.

The equilibrium constant for the Gdn.HCl denaturation of Oval A_1 depends on the denaturant concentration; the equilibrium constant varies with eighth power of Gdn.HCl concentration. The Gdn.HCl denaturation of other proteins (59) is known to show stronger dependence on Gdn.HCl concentration. More exact relation describing this dependence should involve activity of Gdn.HCl, a_g, instead of concentration. Following Wyman (90,91), the dependence of K on a_g will be described in terms of the binding of the denaturant to Oval A_1 by the help of the relation

$$\frac{d\ln K}{d\ln a_g} = \Delta\bar{\nu}_g, \qquad (26)$$

where $\Delta\bar{\nu}_g$ represents the difference in the number of Gdn.HCl bound to the denatured and native forms of Oval A_1. The value of $\Delta\bar{\nu}_g$ was computed to be 6. For other proteins $\Delta\bar{\nu}_g$ roughly correlates with chain length (92). The value of $\Delta\bar{\nu}_g$ for Oval A_1, on the other hand, is the same as that reported for cytochrome c (97) whose chain length is about one-third of that of Oval A_1. It should be noted that Equation 14 would describe the effect of Gdn.HCl on denaturation equilibria in a completely general way so long as the activities of Gdn.HCl and water are independently variable. But this is not true. Protein molecules are hydrated to different extents in the native and denatured states. Therefore, the term, $d\ln K/d\ln a_g$, would yield a value for the preferential binding of Gdn.HCl which will be less in presence of pronounced water binding. It is to be noted that the concentration dependence of the equilibrium constant is most appropriately described by Equation 14 which yields 12 for the value of Δ n. This would imply that the denatured Oval A_1 binds 12 molecules of Gdn.HCl more than the native form.

2. Nature of the Binding Sites.

The present data on Gdn.HCl denaturation of Oval A_1 as well as those available in the literature do not indicate unequivocally

the nature of the binding sites on the protein molecule. From solubility studies of model compounds such as acetyltetraglycine ethyl ester, Robinson and Jencks (94) concluded that Gdn.HCl molecules were bound to the peptide groups. The number of the peptide groups of Oval A_1 which would bind one molecule of Gdn.HCl will be about 30. This appears to be unlikely. Since aromatic amino acids are more soluble in Gdn.HCl solution (95), it is possible that aromatic amino acid residues may offer binding sites for the denaturant. This by implication would mean that, on denaturation, 13 sites became available on the unfolded Oval A_1. Most of the newly exposed binding sites on the denatured Oval A_1 could be tyrosine residues since all the tyrosine residues are inaccessible to solvent in the native state (13-16).

3. The Stability of Native Oval A_1 in the Absence of Gdn.HCl.

The value of log K extrapolated to zero activity of Gdn.HCl according to Equation 14 comes out to be -4.34 which corresponds to a value of 6 Kcal/mole for $\Delta G^{o}_{H_2O}$ at 25°. Thus, the native state of Oval A_1 is favoured over the denatured state only by 6 Kcal/mole. It should be emphasized that several studies on protein denaturation suggest that the native protein conformation is only marginally more stable than the denatured form (96). Considerable part of the instability comes from the large conformational entropy which would stabilize the disordered or denatured protein conformation.

From the results of Figure 11, it is clear that decrease in pH favours the Gdn.HCl induced transition of Oval A_1. From the pH dependence of the equilibrium constant, it was possible to conclude that at least one carboxyl group had a different pH value in the native and denatured states. Since a complete analysis of the data according to Equation 16 could not be achieved, the unequivocal solution for the carboxyl group in question could not be made.

B. Effect of Temperature on Denaturation

That increase in temperature favours Gdn.HCl denaturation of Oval A_1 is evident from Figure 14. As expected, the transition temperature was lower at higher Gdn.HCl concentration. The thermal transition of Oval A_1 was found to be reversible and involved one step at different Gdn.HCl concentrations used in these studies. Oval A_1 is resistant to heat up to 50° (see Figure 13). The effect of temperature on the denaturation appears to be cooperative.

The values of ΔH and ΔS for Gdn.HCl denaturation of Oval A_1 showed temperature dependence as shown in Figure 16. From the least squares analysis of the data of Figure 15, the values of ΔH and ΔS were computed by Equations 20 and 21, and are listed in Table IV which also contains the values of F_{max}, ΔG and ΔCp.

The entropy of denaturation arises primarily from (i) change in conformational entropy as well as from (ii) the change in hydro-

TABLE IV

COMPARISON OF THE VALUES OF ΔG, ΔH, ΔS, ΔCp (ALL AT 25°) AND T_{max} FOR Oval A_1 WITH THOSE FOR OTHER PROTEINS

Protein, Reaction	ΔG (Kcal $mole^{-1}$)	ΔH (Kcal $mole^{-1}$)	ΔS (e.u)	ΔCp (cal $mole^{-1}$ deg^{-1})	T_{max} (°C)
Ribonuclease, thermal transition, pH 2.5	1.4	47	153	2000	- 9
Ribonuclease, 3.1 M Gdn.HCl, pH 6.0[a]	- 0.6	31	110	1800	8
Lysozyme, 1 M Gdn.HCl, pH 7.0[b]	7.9	22	49	1375	13
Chymotrypsinogen, thermal transition, pH 3.0, 1M Cl^-	7.8	39	105	2600	10
Myoglobin, thermal transition, pH 9.0	13.6	42	95	1400	0
β-Lactoglobin, 5 M urea, pH 3.0	0.6	- 21	- 72	2150	35
Oval A_1, 2.6 M Gdn.HCl pH 7.0[b,c]	2.0	53	178	2740	1

Data from reference 58, except:

[a] Reference 96, [b] Reference 97, [c] This study.

phobic side chain during the unfolding exposure. The contribution from the (i) is positive at all temperatures, whereas the contribution from the (ii) may be negative at lower temperature and becomes positive as the temperature is raised. At the temperature of maximum stability, i.e., T_{max} the two contributions balance each other so that ΔS becomes zero. The value of T_{max}, thus, found is near 1^{o} which is similar to that of myoglobin.

The change in enthalpy at 25^{o} for protein denaturations has been reported to be -21 - 47 Kcal/mole; the value of ΔH for the Gdn.HCl denaturation of Oval A_1 is about 53 Kcal/mole. Thus, the Gdn.HCl denaturation of Oval A_1 appears to be highly endothermic process; the value of ΔH is more positive than those hitherto reported for protein denaturations (see Table IV). The Gdn.HCl transition of Oval A_1 is accompanied by a relatively large change in entropy; the entropy of the product of denaturation being more positive than that for the native state. Like other protein denaturations reported so far, the most striking feature of the Gdn.HCl denaturation of Oval A_1 is the large and positive ΔCp. The values of ΔH are sometimes positive and sometimes negative. The positive ΔCp arises because of the unfavourable contacts of hydrophobic moieties to the aqueous solvent. The ordering of the water molecules about hydrophobic groups is sensitive to heat and it is this sensitivity which is responsible for the large anomalous heat capacity. This contention is supported by the elegant studies by Edsall (98), Bohon and Claussen (99) and by Dunn and co-workers (100,101). One of the consequences of large and positive ΔCp is

to make ΔH and ΔS strongly dependent on temperature. At low temperature the ordering of water molecules about non-polar groups would involve a small decrease in enthalpy and relatively large negative entropy (102). The value of ΔCp will tend to make both ΔH and ΔS more positive with increase in temperature. Finally it should be noted that ΔCp for Gdn.HCl denaturation of Oval A_1 depends somewhat on temperature as revealed by computer analysis of the data on temperature dependence of K. For example, the value of ΔCp computed from Equation 25 at 25° is 2,700 cal $mole^{-1}$ deg^{-1} which increases to 4,100 cal $mole^{-1}$ deg^{-1} at 55°. Such positive dependence of ΔCp has been noted for other protein denaturation as well (88,89). It should, however, be noted that the positive temperature dependence of ΔCp noticed in this study can very well be attributed to the experimental uncertainty. Thus, the value of ΔCp varies from the average value (2,700 cal $mole^{-1}$ deg^{-1}) by 10% in the temperature range $15-35^{\circ}$. This variation is understandable in view of the fact that ΔCp is the second derivative of equilibrium constant and even a small uncertainty in the determination of K is expected to be magnified in the estimated value of ΔCp.

C. Kinetics of Denaturation and Renaturation

Results on the kinetics of Gdn.HCl denaturation of Oval A_1 suggested that the transition was reversible and involved one step

as shown by the following discussion. The plot of $\ln|\eta_{Rt=t} - \eta_{Rt=\infty}|$ versus t/t 0.5 was linear in the Gdn.HCl concentration range 1.8 - 3.1 M at 25°. The Gdn.HCl denaturation of Oval A_1 at other temperatures, namely, 15, 35 and 45° followed a first order kinetics. The values of η_R extrapolated to t = 0 for forward and reverse reactions were the same as those for the native and denatured states. Further, the value of η_R extrapolated to t = ∞ was identical to that found during the isothermal denaturation of Oval A_1 (see Figure 6). Furthermore, the rate constants for the forward and reverse reactions depend rather strongly on Gdn.HCl concentration.

It should be noted that the kinetic study of Schellman et al. (73) suggested higher order for the denaturation of Ovalbumin. This can be attributed to the following. First, the ovalbumin preparation used by Schellman et al. was of questionable purity. Second, since whole ovalbumin contains three components, namely, A_1, A_2 and A_3, the latter may undergo denaturation in an independent fashion. Third, the concentration used in their study was about 3-fold higher than that employed in this study. Finally, as evident from the solubility behaviour in Gdn.HCl, Oval A_1 is different from the whole ovalbumin, the latter was insoluble below 2 M Gdn.HCl while the former could be dissolved in as low Gdn.HCl concentration as 0.2 M.

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